Characterization of Expression and Functional Role of Endothelin-1 and its Receptors in Human Lung Fibroblasts

Dissertation

zur

Erlangung des Doktorgrades (Dr.rer.nat.)

der

Mathematisch-Naturwissenschaftlichen Fakultät

der

Rheinischen Friedrich-Wilhelms-Universität Bonn

vorgelegt von

Ahmedat Salih Ahmedat

aus

Misurata-Libyen

Bonn 2014

Angefertigt mit Genehmigung der Mathematisch-Naturwissenschaftlichen
Fakultät der Rheinischen Friedrich-Wilhelms-Universität Bonn

1. Gutachter: Prof. Dr. med. Kurt Racké
2. Gutachter: Prof. Dr. med. Klaus Mohr
Tag der Promotion: 13.05.2015
Erscheinungsjahr: 2015

To my Parents, Family

and those who supported me in any

respect during the completion of this project

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Abbreviations

A

ADP	Adenosine diphosphate
ALK	Activin receptor like kinases
ATP	Adenosine triphosphate

B

BAL	Bronchoalveolar lavage
BMPs	Bone morphogenetic proteins
BSA	Bovine serum albumin

C

Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CBD	cAMP-binding domain
CDC25HD	CDC25-Homology Domain
cDNA	Complementary Deoxyribonucleic acid
CNB	Cyclic-nucleotide-binding
COPD	Chronic obstructive pulmonary disease
CTGF	Connective tissue growth factor

D

DAG	Diacyl glycerol
DEP	Dishevelled-Egl-10-Pleckstrin
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid

E

ECE	Endothelin converting enzyme
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetate
EMT	Epithelial-mesenchymal transition
EndMT	Endothelial-mesenchymal transition

Epac	Exchange proteins activated by cAMP
ERK	Extracellular signal regulated kinase
ET	Endothelin
ET-A	Endothelin receptor A
ET-B	Endothelin receptor B

F

FCS	Fetal calf serum

G

GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GAPs	GTPase Activating Proteins
GDIs	Guanine nucleotide dissociation inhibitors
GDP	Guanosine diphosphate
GEFs	Guanine nucleotide exchange factors
GM-CSF	Granulocyte-macrophage colony stimulating factor
GPCR	G-protein-coupled receptor
G-protein	Guanine nucleotide binding proteins
GTP	Guanosine Triphosphate
GTPase	Guanosine triphosphatase

I

IL	Interleukin
IP3	Inositol triphosphate
IPF	Idiopathic pulmonary fibrosis

J

JNK	Jun N-terminal kinases

L

LPA	Lysophosphatidic acid
LTBP	Latent transforming growth factor binding protein
L-TGF-β	Latent TGF-β

M

MAPK	Mitogen activated protein kinase
MEK	MAPK kinase
MEM	Minimal essential medium eagle
MMP	Matrix metalloproteinases
mRNA	Messenger RNA

N

NEAA	Non essential amino acids
NEP	Neutral endopeptidase enzyme
NO	Nitric oxide

P

P38MAPK	p38 Mitogen-activated protein kinase
PBS	Phosphate buffered saline
PhLFb	Primary human lung fibroblasts
PI	Proteases inhibitors
PI3K	Phosphatidylinositol 3 Kinase
PIP2	Phosphotidylinositol-4,5-biphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PMSF	Protease inhibitors phenylmethansulfonylfluorid
ppET	Prepro-Endothelin
PTX	Pertussis toxin
PVDF	Polyvinylidene difluoride

Q

qPCR	Quantitative real time polymerase chain reaction

R

Raf	Rapidly accelerated fibrosarcoma
Rap	Ras-related protein
Ras	Rat sarcoma

REM	Ras exchange motif
Rho	Ras homologous
RIPA	Radio immuno precipitation assay
RNA	Ribonucleic acid
Rpm	Revolutions (or rotations) per minute
RT	Reverse transcription
RTK	Receptor tyrosine kinase

S

SAPK	Stress-activated protein kinases
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfatepolyacrylamide
SEM	Standard error of the mean
α-SMA	Alpha smooth muscle actin
SPS	Sodium pyruvate solution

T

TGF-β	Transforming growth factor-β
TβR	TGF-β receptors
TNF-α	Tumor necrosis factor-α

V

VEGF	Vascular endothelial growth factor

1. Introduction

Endothelins (ETs) are a family of biologically active peptides, of which endothelin-1 (ET-1) is the best characterized and the most biological relevant isoform in humans. ET-1 is a powerful vasoconstrictor and bronchoconstrictor and mitogenic peptide which can be synthesized and released by different pulmonary cells, among them pulmonary fibroblasts and airway epithelial cells. Endothelinergic system has been involved in multiple physiological function relating to the respiratory system (Hay *et al.*, 1993), but it is also considered to have a potentially pathogenic effects and appears to be implicated in the pathogenesis of various lung disease like pulmonary hypertension, pulmonary fibrosis and fibrotic remodeling processes associated with chronic inflammatory and obstructive airway diseases such as bronchial asthma and chronic obstructive pulmonary disease (COPD) (Hay, 1999; Fagan *et al.*, 2001; Polikepahad *et al.*, 2006). Thus, the endothelinergic system emerged as a potential target for pharmacological intervention in the treatment of these diseases. These pathological conditions are characterized mainly by increased growth of connective tissue cells and excessive synthesis and secretion of matrix proteins, resulting in an increased extracellular matrix (ECM) deposition that lead to significant functional limitations of the lung tissue. Pulmonary fibroblasts are connective tissue cells and are the main cells responsible for the synthesis, deposition and remodeling of ECM in tissues. Thus, they significantly participate in pathophysiologic events of airway remodelling. Therefore, endothelinergic mechanisms in human lung fibroblasts are in the focus of the present study.

1.1. Role of fibroblasts in airway remodeling process

1.1.1. Lung fibroblasts

Fibroblasts are connective tissue cells of mesenchymal origin that are widely distributed in all lung structures. They represent the most abundant cell type in the lung interstitium. Fibroblasts are typically spindle-shaped cells with an oval flat nucleus, but they do not express markers of other differentiated cell types. They are ubiquitous cells that provide mechanical strength to tissues by generating ECM components which form a supporting framework. Fibroblasts are considered to be the main cells responsible for the synthesis, deposition and remodelling of ECM. The ability of fibroblasts to respond to many different mediators which they release or which are produced by other resident cells like epithelial and endothelial cells, as well as mediators derived from circulation allows autocrine and/or reciprocal paracrine interactions, thereby maintaining tissue homeostasis (Kalluri *et al.*, 2006; Laurent *et al.*, 2007). These interactions control the morphogenesis of tissue structures and accordingly, fibroblasts play a crucial role during tissue development, differentiation and repair in many organs including lung (Phan, 2008; Westergren-Thorsson *et al.*, 2010). In addition, there is evidence that in fibrotic disease like idiopathic pulmonary fibrosis (IPF), ET-1, transforming growth factor-β (TGF-β), platelet-derived growth factor and connective tissue growth factor (CTGF) are capable of promoting fibroblasts activation, migration and proliferation as well as accumulation (Datta *et al.*, 2011).

Fibroblasts in normal tissues exhibit cell-ECM contacts and produce little matrix proteins. They are generally held by cross-linked ECM, but in injured tissue, this links to the ECM are lost (Tomasek *et al.*, 2002). This alteration in microenvironment in turn activates fibroblasts to proliferate as well as to synthesize ECM components (Hinz *et al.*, 2007b). Local over expression of cytokines and/or growth factors like TGF-β and ET-1 during the pathogenesis of lung fibrosis induces resident lung fibroblasts to synthesize elevated levels of ECM components (Datta *et al.*, 2011). Fibroblasts are metabolically active cells, capable of continuously synthesizing and secreting ECM components like collagens and proteoglycans. However, the amount they produce is tightly controlled by secreting enzymes that control ECM degradation including matrix metalloproteinases (MMPs) (Laurent *et al.*, 2007). In addition fibroblasts also produce tissue inhibitors of metalloproteinases,

thus regulating tissue architecture and matrix turnover rates. The main ECM components produced by lung fibroblasts are fibrillar collagens; predominantly type I and III collagens, along with protoglycan and fibronectin. These are important ECM proteins for both, the organizational and structural pulmonary architecture network (Prockop *et al.*, 1995; Westergren-Thorsson *et al.*, 2010).

Lung fibroblasts play an essential role in the repair processes following lung injury. Accordingly, after tissue injury, fibroblasts become activated to migrate into the damaged tissue and are stimulated to synthesize and secret collagen and fibronectin-rich matrix for wound closure (Kuhn *et al.*, 1991; Hinz, 2007a). However, the healing process can become pathological causing continuous activation of the fibroblasts proliferation as well as excessive production and accumulation of interstitial ECM proteins in particular collagen that progressively leads to alterations in tissue architecture with impairment of organ function (Wynn, 2008). This alteration is believed to be a histopathological feature of chronic pulmonary diseases such as bronchial asthma, COPD (Hogg *et al.*, 2004; Bergeron *et al.*, 2006; Rennard *et al.*, 2011) and idiopathic pulmonary fibrosis (Selman *et al.*, 2001).

Human lung fibroblasts are frequently utilized during *in vitro* studies. This is a suitable technique to investigate pro- and anti-fibrotic processes in these cells along with the structural changes at the cellular level. The fibroblasts and in particular lung fibroblasts are not a unique cell population. Fibroblasts exhibit phenotypic heterogeneity and considerable regional variation in size, shape, synthesis performance and their content of alpha-smooth muscle actin (α-SMA) (Kotaru *et al.*, 2006; Zhou *et al.*, 2011). In contrast to other cells types, lung fibroblasts do not have a typical surface marker that allows a definite characterization and classification to subpopulations (Phan, 2008).

1.1.2. Myofibroblasts

Myofibroblasts are fibroblast-like cells, characterized by the expression of α-SMA, the actin isoform typical of smooth muscle cells, conferring a highly contractile activity to these cells (Hinz *et al.*, 2001). The expression of α-SMA is the most commonly used molecular marker for myofibroblast identification. This allow to assessing the differentiation state of fibroblasts into myofibroblasts during experimental and clinical conditions. The morphologic and biochemical characters of myofibroblasts are intermediate between fibroblasts and smooth muscle cells

(Gabbiani, 1992). Myofibroblasts play a central role in producing ECM proteins, including collagen (Hinz *et al.*, 2007b). In comparison with fibroblasts, they synthesize considerably more matrix proteins, thus contributing to the structural remodeling of the connective tissue. Myofibroblasts are also responsible for the synthesis of enzymes involved in ECM degradation such as matrix metalloproteinase 2 (MMP-2) (Desmouliere *et al.*, 2004). The functional activity of myofibroblasts in part proceeds through expression of α-SMA, a protein which promotes wound contraction and thereby contributes to wound healing (Grinnell, 1994; Tomasek *et al.*, 2002). By synthesizing elevated levels of both ECM and matrix degrading components, α-SMA-producing myofibroblasts also promote tissue repair and wound healing (Desmouliere *et al.*, 2005). For normal healing to occur, wound myofibroblasts must undergo apoptosis (Desmouliere *et al.*, 1995a); however, if the tissue repair process does not appropriately terminate (failure of apoptosis), myofibroblasts accumulate at the site of the lesion, resulting in an extensive ECM synthesis, persistent tissue contraction and scar formation (Tomasek *et al.*, 2002), thereby contributing to the development of fibrosis. Myofibroblasts are thought to be a major source of cytokines, chemokines and profibrogenic growth factors (Kuhn *et al.*, 1991; Zhang *et al.*, 1994). Under pathological conditions, excessive production of these factors can lead to lung remodeling and inflammation that may affect lung function (Hinz *et al.*, 2007b). Myofibroblast numbers were found to be elevated in the airways of asthmatic subjects (Brewster *et al.*, 1990; Gabbrielli *et al.*, 1994; Holgate *et al.*, 2000).

Myofibroblasts can originate from several cell types, including resident fibroblasts (Phan, 2002), circulating fibrocytes attracted to regions of lung injury (Schmidt *et al.*, 2003; Lama *et al.*, 2006), endothelial-mesenchymal transition (EndMT) (Hashimoto *et al.*, 2010) and epithelial-mesenchymal transition (EMT) (Willis *et al.*, 2005; Kim *et al.*, 2006). However, it is postulated that resident fibroblasts are the most obvious source of myofibroblasts in lung fibrosis (Hinz *et al.*, 2007b).

ET-1 can promote the induction of the myofibroblast phenotype. For example, ET-1 has been demonstrated to transdifferentiate resident fibroblasts into myofibroblasts in various tissues, including the lung (Lagares *et al.*, 2012). In addition, ET-1 can also induce EMT/EndMT in epithelial/endothelial cells as a source

of myofibroblasts in the fibrotic disease (Jain *et al.*, 2007; Piera-Velazquez *et al.*, 2011). Moreover, ET-1 has been shown to be able to promote myofibroblast resistance to apoptosis and thereby may contribute to the persistence of fibrotic response (Horowitz *et al.*, 2012). Furthermore, TGF-β, a main promoter of fibrosis, induces transdifferentiation of fibroblasts *in vitro* through a Smad3-dependent mechanism (Hu *et al.*, 2003).

1.2. Extracellular matrix (ECM)

ECM is a complex network of macromolecules (proteins) that provide structural and biochemical support to the surrounding cells. ECM represents the main part of the connective tissue of the respiratory airways and plays an essential role in determining physical and mechanical properties of airway structure. ECM macromolecules also contribute to a number of cell functions like migration, proliferation, differentiation, survival and communication (Daley *et al.*, 2008). Due to these multiple actions, it is believed that alteration in the ECM structure can cause dramatic effects on tissue homeostasis and may promote progression of disease like tissue fibrosis and cancer. Lung fibroblasts are the main producer of ECM proteins in airways. Composition and structure of the ECM vary considerably in the different types of connective tissues and determine the functional and biological characteristics. ECM protein production is increased by profibrotic factors like TGF-β, CTGF, granulocyte macrophage colony stimulating factor (GM-CSF) and ET-1 (Abraham, 2008; Burgess, 2009). The ECM in airways is subjected to a continuous turnover of more than 10% of the total ECM per day (McAnulty *et al.*, 1988). Thus, a dynamic equilibrium between synthesis and degradation of the ECM maintains the physiological balance (Laurent, 1986; Fernandes *et al.*, 2006). Degradation of ECM is tightly controlled by a diversity of proteases and binding proteins, mainly the MMPs and their inhibitors, the tissue inhibitors of MMPs (TIMPs) and A disintegrin and metalloproteinases (ADAMs) (Parks *et al.*, 2001; Page-McCaw *et al.*, 2007).

Two main classes of extracellular macromolecules make up the matrix (i) polysaccharide chains of the class called glycosaminoglycans (GAGs), which are covalently linked to proteins in the form of proteoglycans. This includes hyaluronic acid, heparan sulfate, keratan sulfate and chondroitin sulfate, and (ii) fibrous proteins, including collagen, elastin, fibronectin and laminin, which have both

structural and adhesive functions. Collagens constitute the main component of the ECM proteins, and are gaining attention from scientists in different fields, ranging from biochemistry to regenerative medicine. As in the present study the collagen production was investigated more closely, collagens as component of the ECM will be described in more detail. Collagens are a family of fibrous proteins, and are the most prevalent proteins in human body, accounting for about 30% of the total protein mass (Myllyharju *et al.*, 2004; Ricard-Blum, 2011) and constitute about 15% of the dry weight of human lung tissue (Laurent, 1986). At least 28 different collagen types have been identified so far, and are numbered as I-XXVIII (Heino, 2007; Shoulders *et al.*, 2009). They are differentiated according to their tissue localization, their molecular structure, their aggregation behaviour in the extracellular space and their function. Classically, collagens can be categorized as fibril-forming collagens and non-fibrillar collagens according to their polymeric structures. However, the non-fibrillar collagens can be further divided into several subgroups based on their supramolecular organization and other characteristics: fibril-associated collagens with interrupted triple helices (FACITs), network-forming collagens, membrane-associated collagens with interrupted triple helices (MACITs), collagens forming anchoring fibrils and collagens with multiple triple-helix domains and interruptions (MULTIPLEXINs) (Gelse *et al.*, 2003; Myllyharju *et al.*, 2004; Veit *et al.*, 2006; Shoulders *et al.*, 2009). In the human body, the most abundant and widespread family of collagens, with about 90% of the total collagen, consists of the fibril-forming collagens (Gelse *et al.*, 2003), which include type I, II, III, V, XI, XXIV, XXVII, existing as fibrils in the ECM. Although the relatively high structural diversity among the different collagen types, all types of collagen have a triple-stranded helical structure composed of three polypeptide chains (α-chains) and are held together to form long thin fibrils arranged in bundles (rope like superhelix), which can be of varying lengths (Gelse *et al.*, 2003; Ricard-Blum, 2011). Collagens are extremely rich in proline and glycine, which are essential in the formation of the triple-stranded helix. Proline, because of its ring structure, stabilizes the helical structure in each α chain, whereas glycine, the smallest amino acid enables the tight packing of the triple helical α chains to form the final collagen superhelix. The hydroxylation of proline by collagen prolyl 4-hydroxylase enzymes further increases the thermal stability of triple helices (Myllyharju, 2008). The long stiff chains contain characteristic repeating peptide triplets of glycine-X-Y with varied length, in which the X-position is often occupied by

proline and the Y-position by hydroxyproline (Prockop *et al.*, 1995; Gelse *et al.*, 2003). Collagens are synthesized as the collagen precursor, procollagen molecules containing N- and C-terminal propeptide extensions. These soluble propeptides are proteolytically cleaved by specific procollagen proteinases during their secretion into the ECM to yield insoluble mature collagen, thereby triggering spontaneous self assembly of collagen molecules into fibrils. The C-terminal propeptides are essential in initiating the association of the α-chains and ensure solubility in the extracellular space, while the N-terminal propeptides influence fibril shape and diameter (Hulmes, 2002).

1.3. Endothelin

1.3.1. Endothelin biology

In 1985, Hickey and co-workers reported the presence of a peptidergic endothelium-derived constricting factor produced by bovine aortic endothelial cells (Hickey *et al.*, 1985). This factor was subsequently isolated from cultured supernatant of porcine aortic endothelial cells, sequenced, cloned and named endothelin (ET), which is known today as endothelin-1 (ET-1). It is a potent vasoconstrictor peptide consisting of 21 amino acid residues (Yanagisawa *et al.*, 1988). Following the discovery of ET-1, analysis of the human gene encoding ET demonstrated the presence of two other genes encoding ET-like peptides and referred to endothelin-2 (ET-2) and endothelin-3 (ET-3), which are each encoded by a separate and unique gene (Inoue *et al.*, 1989). The human genes of ET-1, ET-2 and ET-3 are located on chromosomes 6, 1 and 20, respectively (Arinami *et al.*, 1991). All the three ET isoforms possess 21 amino acids residues with rather similar sequences, but ET-1 differ in their mature structure from ET-2 and ET-3 by two and six amino acid residues, respectively (Fig. 1-1) (Yanagisawa *et al.*, 1988). All of the ET isoforms share a common structure, with two intra-chain disulphide bridges linking the cysteine amino acids (positions 1–15 and 3–11) at the N-terminal end and hydrophobic amino acids at the C-terminal end of the peptide (Fig.1-1). The N-terminal end determines the binding affinity of the peptide to the receptor, whereas the C-terminal end consists of the amino acids that bind to the receptor (Inoue *et al.*, 1989). Each endothelin isoform is expressed in various cell types and tissues with distinct proportions, however ET-1 is the only isoform expressed by endothelial cells (Inoue *et al.*, 1989; Sakurai *et al.*, 1991).

Among all ET isoforms, ET-1 is the best characterized, the most potent vasoconstrictor peptide, the most abundant and physiologically relevant isoform in humans and may exert both beneficial and detrimental effects (Kedzierski *et al.*, 2001; Abraham, 2008). Therefore, ET-1 is in the focus of the current thesis.

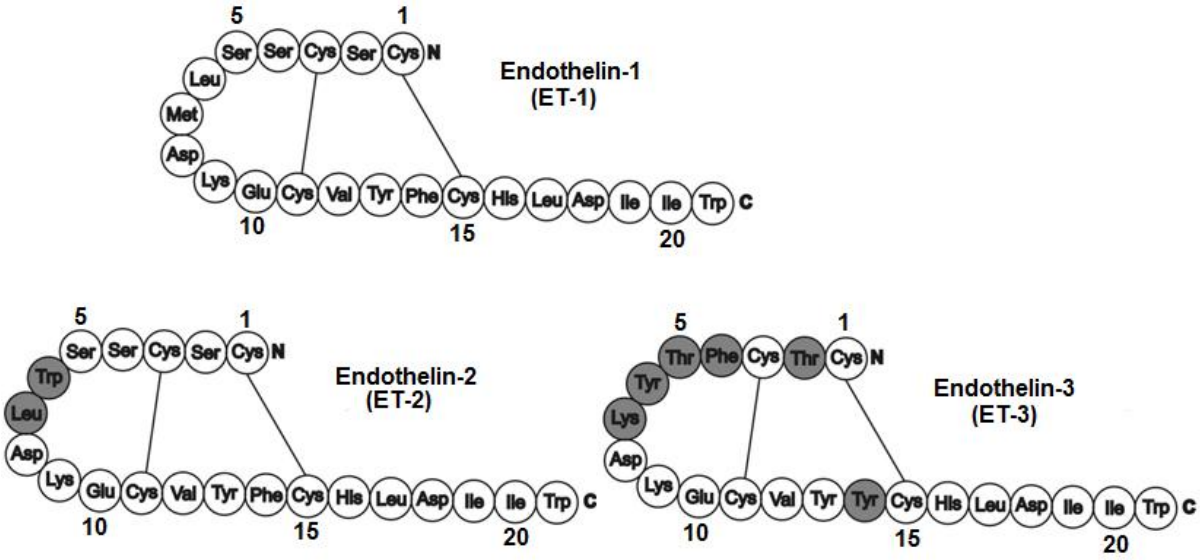


Figure 1-1: Primary sequences of the three ET isoforms. Amino acids that differ from the ET-1 sequence in ET-2 and ET-3 are shown in grey. Adapted from (Kedzierski *et al.*, 2001).

1.3.2. Biosynthesis of endothelin

Although the endothelial cells are considered the main physiological source of ET-1 in humans (Boscoe *et al.*, 2000), many other cell types can synthesize and release ET-1, including epithelial cells, fibroblasts, macrophages, vascular smooth muscle cells, cardiomyocytes (Kedzierski *et al.*, 2001) implying numerous potential roles for ET-1 in various aspects of human physiology, pathology, and pharmacology. ET-1 synthesis requires several processing steps before the mature peptide is formed. The biosynthesis of all three endothelin isoforms (ET-1, ET-2 and ET-3) follows a similar pattern. ETs synthesis is initiated by the transcription of the prepro-endothelin (ppET) genes (Fig.1-2). The translation of ppET mRNA results in the generation of a 212 amino acid ppET peptide, a large inactive precursor of ET peptides. The precursor ppET is proteolytically cleaved within the cell at dibasic sites by a furin-like endopeptidase enzyme to a 38 (human) or 39 (porcine) amino acid intermediate molecule, referred to as big endothelin (big ET) or proendothelin (pET) (Inoue *et al.*, 1989). pET-1 has some vasoconstrictive activity. It displays about two

orders of magnitude less vasoconstrictor potency than the active ET-1 (Kashiwabara *et al.*, 1989; D'Orleans-Juste *et al.*, 2003). Each of proendothelins (pET-1, pET-2 and pET-3) is indiscriminately further cleaved at Trp_{21}-Val_{22} bond by an endothelin converting enzyme (ECE), a membrane-bound zinc metalloendoprotease, which cleaves 17 (human) or 18 (porcine) amino acids from the C-terminus of big ET to generate its corresponding 21 amino acid, bioactive ET peptide (Fig.1-2) (Kedzierski *et al.*, 2001; Masaki, 2004).

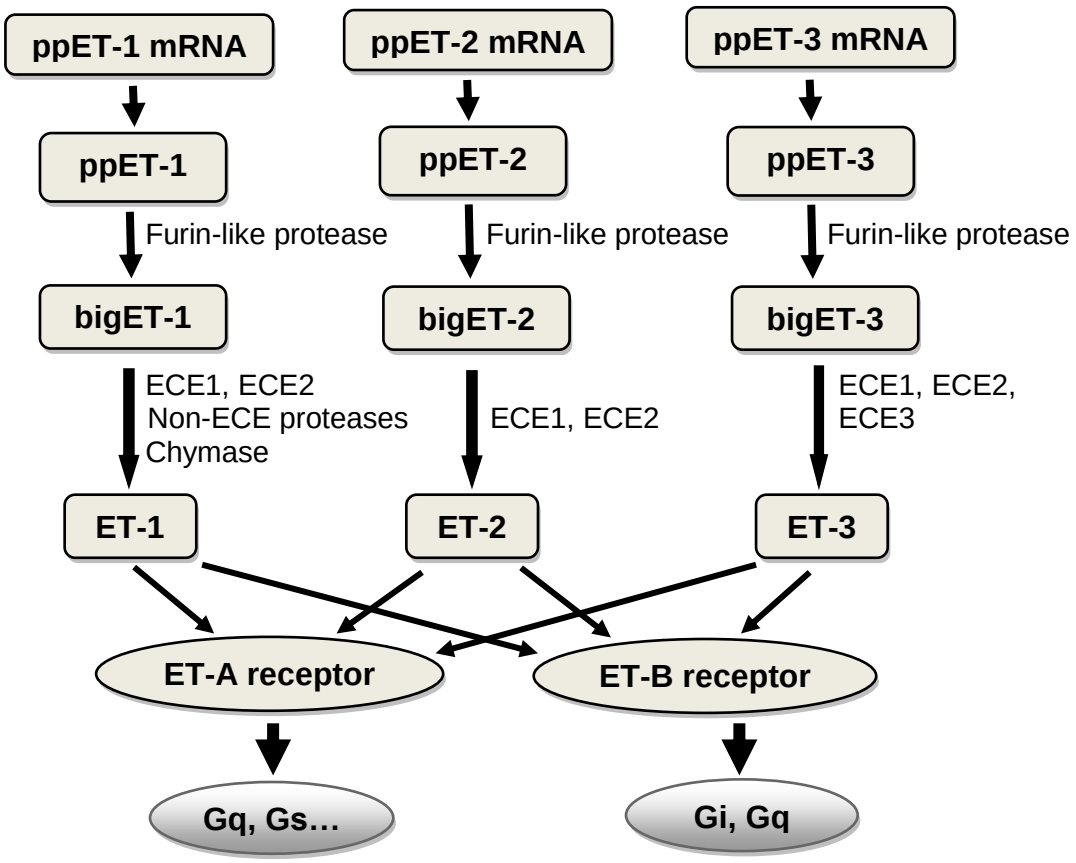


Figure 1-2: Schematic diagram of the endothelins biosynthesis. ppET, prepro-endothelin; ECE, endothelin converting enzyme; ET-A, endothelin receptor-A; ET-B, endothelin receptor-B.

1.3.3. Endothelin converting enzyme (ECE)

ECEs are membrane-bound zinc metallopeptidases that catalyze the final step in the ppET activation to the bioactive ET. Three isoforms of ECE have so far been described known as ECE-1, ECE-2 and ECE-3, with different specificities for the isoforms of big ET (D'Orleans-Juste *et al.*, 2003). ECE-1 and ECE-2 are most prominent (Emoto *et al.*, 1995), and can generate all three ETs isoforms. However, ECE-1 is the major functional converting enzyme ECE for all three ET isoforms (Emoto *et al.*, 1995; Yanagisawa *et al.*, 2000). It exists as a dimer on the cell surface

(Xu *et al.*, 1994). Both ECE-1 and ECE-2 display preference for big ET-1 over big ET-2 or big ET-3.

Four functionally distinct isoforms of human ECE-1, (ECE-1a-d) have been identified to date (Shimada *et al.*, 1995; Valdenaire *et al.*, 1995; Schweizer *et al.*, 1997; Hunter *et al.*, 2006), with similar enzymatic properties and identical efficiency. They are derived from alternative splicing of a single gene but, there are subtle differences amongst the four isoforms in the cytoplasmic N-terminus, resulting in distinct cellular localization (Schweizer *et al.*, 1997; Jafri *et al.*, 2003). The different localization of ECE-1 isoforms means that ECE-1 can convert big ETs to the bioactive ET both intracellularly and extracellularly. These isoforms of ECE-1 display similar specific activity and identical efficiency for conversion of big ET-1 to the bioactive ET-1, but are less efficient for conversion of big ET-2 and big ET-3.

ECE-2 shares 59% identity with ECE-1, and converts ETs with about similar efficiency as ECE-1. ECE-2 also consists of four isoforms with varying N-terminus that may confer different intracellular, but no extracellular localization (Emoto *et al.*, 1995; Ikeda *et al.*, 2002). ECE-2 has an acidic optimal pH of 5.5, with virtually no activity at neutral pH 7.0 (Emoto *et al.*, 1995), suggesting that ECE-2 acts as an intracellular enzyme and particularly in association with the trans-Golgi network, in which the pH is acidic (Emoto *et al.*, 1995).

ECE-3 was isolated from bovine iris microsomes, in which ET-3 concentrations are higher than those of ET-1 (Hasegawa *et al.*, 1998). It has preference for big ET-3 rather than for big ET-1 (Hasegawa *et al.*, 1998).

In addition, non-ECE proteases such as chymase, a mast cell-derived serine protease and MMP-2 could also be involved in the cleavage of bigET-1 to a 31 and 32 amino acid intermediate ET-1 peptides respectively, that can subsequently be cleaved by a neutral endopeptidase enzyme (NEP) and/or ECE to the biologically active ET-1 (Nakano *et al.,* 1997; Honore *et al.,* 2002; D'Orleans-Juste *et al.*, 2003; Fecteau *et al.*, 2005; Simard *et al.*, 2009).

1.3.4. Regulation of endotheline-1 (ET-1) expression and secretion

The expression of ET-1 is regulated primarily at the mRNA level that involves both transcriptional and posttranscriptional mechanisms (Inoue *et al.*, 1989; Rodriguez-Pascual *et al.*, 2003; Mawji *et al.*, 2004). The ET-1 mRNA is relatively

labile with an intracellular half life of less than an hour (Bloch *et al.*, 1989; Inoue *et al.*, 1989), which is partly due to the presence of three destabilizing AUUUA motifs (suicide motifs) in the 3'-untranslated region (Inoue *et al.*, 1989). The intrinsic mRNA instability confers on the ET-1 gene the capacity to respond quickly to changes in transcriptional activity. Thus, the combined actions of ET-1 transcription and rapid mRNA turnover allow for stringent control over its expression. In fact, alteration in ET-1 synthesis has been demonstrated in some studies that can be affected by modification of ET-1 mRNA stability (Douthwaite *et al.*, 2003; Mawji *et al.*, 2004; Rodriguez-Pascual *et al.*, 2008). ET-1 is not stored but instead produced and released in response to various stimuli, which differ between different tissues (Haynes *et al.*, 1998). The expression of ET-1 gene, ET-1 synthesis and release are elevated in response to physiological and pathological stimuli such as shear stress, hypoxia, catecolamines, angiotensin II, cytokines like TGF-β and tumor necrosis factor-alpha (TNF-α), interleukins and other growth factors (Teder *et al.*, 2000; Kedzierski *et al.*, 2001). On the other hand, ET-1 synthesis in endothelial cells is inhibited by nitric oxide, prostacycline, heparin and natriuretic peptide (Kedzierski *et al.*, 2001). Plasma levels of ET-1 in the circulation of healthy individuals are in the picomolar range (1–10 pmol/L), lower than those required for many of the biological actions of ET-1 (Haynes *et al.*, 1998). Thus, the circulating ET-1 levels might not correspond to the full biological ET-1 effect. Therefore, under normal physiological conditions ET-1 is considered to function in a paracrine/autocrine manner at various sites in the body (Kedzierski *et al.*, 2001) rather than as circulating hormone (Wagner *et al.*, 1992; Haynes *et al.*, 1998).

1.3.5. ET-1 clearance

Although the biological effects of ET-1 may last considerably longer, the plasma clearance of circulating ET-1 is very rapid, with a half life of less than two minutes (Gasic *et al.*, 1992), which may explain the low plasma levels of ET-1. In the rat, the half life as determined by intravenously injected, labelled ET-1 was less than one minute, whereas the pressor effects were maintained for about one hour (Sirvio *et al.*, 1990). Nonetheless, levels of ET-1 in the plasma reflect a balance between its production and clearance (Kirkby *et al.*, 2008). ET-1 appears to undergo either receptor- or enzyme-mediated degradation. ET-1 is mainly cleared by ET-B receptor-

mediated degradation, and is in addition degraded by NEP. The lung act as the main site for production (Dupuis *et al.*, 1996b) and clearance of circulating ET-1 (Anggard *et al.*, 1989). In a single passage through the lung, about 50% of circulating ET-1 is cleared, but about the same amount of ET-1 is newly released into the circulation (Dupuis *et al.*, 1996b). The lung in addition plays a major target for ET-1 effects. In the rat, the highest levels of ET-1 mRNA and immunoreactive ET-1 has been found in the lung in comparison with other organs (Matsumoto *et al.*, 1989). ET-1-like immunoreactivity and mRNA expression have been demonstrated also in healthy human lung tissue (Giaid *et al.*, 1993).

1.3.6. Endothelin receptors

The downstream effects of ET-1 are mediated by two distinct receptor subtypes termed ET-A receptor (Arai *et al.*, 1990) and ET-B receptor (Sakurai *et al.*, 1990). These receptors are members of the rhodopsin class A seven transmembrane guanine nucleotide-binding protein coupled receptor (GPCR) subfamily, which mediate various cellular events by activation of heterotrimeric guanine nucleotide binding proteins (G proteins). The human ET-A and ET-B receptors are encoded by distinct genes located on chromosomes 4 and 13, respectively (Sakurai *et al.*, 1992). The human ET-A receptor consist of 427 amino acids, and shares about 64% sequence homology with the estimated 442 amino acids human ET-B receptor (Adachi *et al.*, 1991). Both receptors have seven trans-membrane domains of 22-26 hydrophobic amino acids residues with an extracellular N-terminus and a cytoplasmic (intracellular) C-terminus. (Arai *et al.*, 1990; Sakurai *et al.*, 1990). Although they share high sequence similarity, the activated ET-A and ET-B receptors have quite different signalling transduction and targeting mechanisms (Aramori *et al.*, 1992; Takimoto *et al.*, 1996), and accordingly may mediate different responses. They exhibit distinct affinities for the different ET isoforms. The ET-A receptor binds ET-1 and ET-2 with greater affinity than ET-3, whereas the ET-B receptor binds all the three ET isoforms (ET-1, ET-2 and ET-3) with similar affinity (Sakurai *et al.*, 1992). Therefore, ET-B receptor is considered as a non isopeptide selective receptor. There are multiple agonists recognized by the ET-B receptor, including sarafotoxin 6c and IRL1620 (Watts, 2010), but up to date, no selective agonists have been described for the ET-A receptor. Nevertheless, selective

competitive peptide and nonpeptide antagonists for ET-A as well as ET-B receptors proved to be important tools to study the function of each receptor type (Davenport, 2002).

Both ET receptors (ET-A and ET-B) are widely distributed in a variety of human tissues and cells including lung, heart, kidney and brain, but with different expression levels (Davenport *et al.*, 1989; Jones *et al.*, 1989). The distribution and expression levels of ET receptors varies among different organs and tissues, and may also dependent on the developmental stage and change during pathological conditions (Frommer *et al.*, 2008). The density of ET receptors binding sites is particularly high in the lung and heart (Simonson *et al.*, 1990). ET-A receptors in normal lungs are predominantly expressed on mesenchymal cells such as vascular and airway fibroblasts and smooth muscle cells (Fagan *et al.*, 2001; Abraham, 2008). ET-B receptors, although mainly expressed on the endothelium (Fagan *et al.*, 2001), are also expressed on other mesenchymal cells (Abraham, 2008). Both receptors (ET-A and ET-B) often act synergistically, but can also modulate some functions independently of one another and exhibit somewhat different physiological roles. For instance, ET-1-induced activation of ET-A receptors on smooth muscle cells causes vasoconstriction, cell growth and cell adhesion. Conversely, binding of ET-1 to endothelial ET-B receptors leads to vasodilation through the release of nitric oxide (NO) and prostacycline which also exhibit anti-proliferative properties, inhibits ECE-1 expression and prevents apoptosis (D'Orleans-Juste *et al.*, 2002; Alonso *et al.*, 2003). Importantly, ET-B receptors on endothelial cells are involved in the clearance of circulating ET-1 (Dupuis *et al.*, 1996b). In some tissues the ET-B receptors mediate vasoconstriction and cell proliferation, thus both receptor subtypes implicated in the profibrotic effects of ET-1 (Clozel *et al.*, 2005; Clozel *et al.*, 2006).

1.3.7. Endothelin receptor-mediated signaling pathways

Given that ET receptors belong to GPCRs, their signal transduction was initially interpreted as sequential series of events initiated by binding of the agonist to its receptor, leading to activation of a heterotrimeric G protein which in turn is linked to either stimulatory or inhibitory second messenger pathways. The resulting change in second messenger concentration then leads to further downstream effector events, frequently activation of protein kinases. G-proteins transduce various cellular effects, including enzyme activation/deactivation, downstream gene transcription,

protein synthesis, and cell division. The heterotrimeric G-proteins are composed of three subunits, designated alpha, beta, and gamma (α, β and ϒ), and are classified according to their alpha subunit. Therefore, G proteins in general are referred to their Gα-subunit. So far, at least 23 distinct α-, 5 β- and 12 ϒ-subunits have been identified. G proteins are typically divided into four main subfamilies based on their amino acid sequence similarity and coupling specificity of the Gα subunit, namely $G\alpha_s$, $G\alpha_i$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$ (Simon *et al.*, 1991). Each Gα sub-family activates a specific profile of effectors. G-proteins change between their active and inactive state by binding guanosine triphosphate (GTP) or guanosine diphosphate (GDP) nucleotides. In the inactive state, the α-subunit of the G protein binds GDP, the Gα-GDP is tightly associated with the Gβγ-complex, and this heterotrimeric is attached to the cytoplasmic surface of the plasma membrane (Fig.1-3). When bound to GTP, G protein is in the active state and can activate directly different effector molecules. The intrinsic GTPase activity of Gα leads to hydrolysis of GTP to GDP and the re-association of Gα-GDP and Gβγ subunits with termination of signalling (Fig.1-3). The stimulation of the ET receptors induces the exchange of the GDP to GTP. The binding of GTP leads to dissociation of βγ-complex from α-subunit, consequently both subunits can activate distinct downstream intracellular effectors (Hamm, 1998).

GPCRs downstream pathways have been divided into two sub-groups, pertussis toxin- (PTX) sensitive (coupled to $G_{i/o}$) or PTX-insensitive (coupled to $G_{q/11}$, G_s, or $G_{12/13}$) (Albert *et al.,* 2002; Pierce *et al.,* 2002). PTX, an exotoxin produced by Bordetella pertussis, has been widely used as a pharmacological tool to characterize the involvement of heterotrimeric G-proteins in signalling. This bacterial toxin catalyses the ADP-ribosylation of the α subunits of the G_i family, and thereby inhibit G_i-mediated signalling (Bokoch *et al.*, 1983). Both ET receptors are capable of coupling to several G proteins, including $G\alpha_i/G\alpha_o$ (PTX-sensitive), $G\alpha_q/G\alpha_{11}$ and $G\alpha_{11/12}$ (PTX-insensitive) as well as $G\alpha_s$, although the coupling efficiency of each receptor subtype to G proteins may be different depending on the expression level of each G protein subtype in the cell (Aramori *et al.*, 1992; Takigawa *et al.*, 1995; Arai *et al.*, 2003). This suggests that the downstream signaling may differ depending on the G protein involved (Aramori *et al.*, 1992; Takigawa *et al.*, 1995). Thus, the overall effects induced by ET-1-mediated activation of ET receptors may differ in various cell types. The PTX-insensitive coupling to phospholipase C (PLC) is mediated by $G\alpha_q$ and $G\alpha_{11/12}$, whereas the PTX-sensitive coupling to adenylyl cyclase inhibition is

mediated by $G\alpha_i$, $G\alpha_o$, $G\alpha_{i1}$, $G\alpha_{i2}$ and $G\alpha_{i3}$. Adenylyl cyclase converts adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP).

Downstream signalling of ET-A receptor is primarily mediated by the activation of G-proteins of the $G\alpha_{q/11}$ subfamily, resulting in activation of PLC that cleaves phosphotidylinositol-4,5-bisphosphate (PIP2) into inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) (Aramori *et al.*, 1992; Eguchi *et al.*, 1993b). IP_3 and DAG are two second messengers that activate the release of Ca^{2+} and protein kinase C (PKC), respectively, promoting further signaling cascades. ET-A receptor can also couple to $G\alpha_s$ resulting in activation of adenylyl cyclase and cAMP production (Takagi *et al.*, 1995).

ET-B receptors can also couple to $G\alpha_{q/11}$, but in contrast to ET-A receptors, they predominantly couple to $G\alpha_{i/o}$, $G\alpha_{i3}$ (Aramori *et al.*, 1992; Eguchi *et al.*, 1993b; Gohla *et al.*, 1999). Accordingly, activation of the ET-B receptors is associated with inhibition of cAMP formation as well as activation of PIP2 hydrolysis and production of IP_3 and DAG. (Eguchi *et al.*, 1993a). Thus, the two ET receptor subtypes couple to multiple but distinct signal transduction.

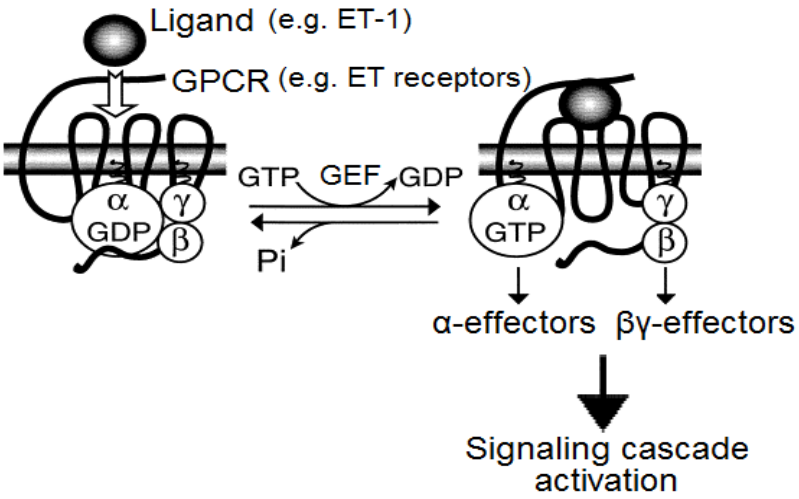


Figure 1-3: Schematic diagram of the guanine nucleotide cycle governing G protein coupled receptor (GPCR)-mediated activation of heterotrimeric G protein-coupled signaling. ET-1, endotheline-1; GDP, Guanosine diphosphate; GEF, Guanine nucleotide exchange factor; GTP, Guanosine Triphosphate.

1.4. Adrenergic and cholinergic system of the airways

Human airways are innervated by efferent and afferent autonomic nerves which control various features of airway function. The innervations of the lung can be

divided into cholinergic and adrenergic pathways, along with non-adrenergic non-cholinergic pathways. It is well recognized that human lung fibroblasts are under the regulation of both adrenergic and cholinergic (acetylcholine) mechanisms, mediated via β-adrenoreceptor and by muscarinic receptors, respectively. Based on their bronchodilatory effects, β_2-adrenergic agonists and anticholinergic drugs represent major components in the treatment of bronchial asthma and/or COPD. However, the action of these drugs is not confined to bronchodilation (Racké *et al.*, 2008; Bateman *et al.*, 2009). Thus, it was recently demonstrated in our laboratory that pro-fibrotic features such as proliferation and collagen synthesis in human lung fibroblasts are stimulated by muscarinic receptors (Matthiesen *et al.*, 2006; Haag *et al.*, 2008a), but inhibited by β_2-adrenoceptors (Lamyel *et al.*, 2011). Therefore, in one part of this study, a possible regulation of the ET-1 gene expression in human lung fibroblasts by cholinergic and/or adrenergic mechanisms was investigated.

1.4.1. β_2–Adrenoceptor signaling

Three subtypes of β-adrenoreceptors have so far been identified (β_1, β_2 and β_3) (Bylund *et al.*, 1994). They belong to the GPCR Rhodopsin family, which are encoded by three different genes located on human chromosomes 10, 5 and 8, respectively. Among these three subtypes, the β_2-adrenoceptor is widely distributed in various human tissues and cells (Barnes, 1995). Within the human lung, β_2-adrenoceptors but not β_1- or β_3- adrenoceptors are expressed in lung fibroblasts, epithelial, and smooth muscle cells (Mak *et al.*, 1996; Lamyel *et al.*, 2011). Generally, β_2-adrenoceptors couple to the stimulatory G proteins ($G\alpha_s$), resulting in cAMP production. However, they are also able to interact with other G protein families, such as the inhibitory $G\alpha_i$ and $G\alpha_q$ family (Rubenstein *et al.*, 1991; Wenzel-Seifert *et al.*, 2000).

Adenosine 3′,5′ cyclic monophosphate (cAMP) has long been identified as signal transduction molecule, acting as second messenger in response to a variety of extracellular signals (Beavo *et al.*, 2002). The cAMP signaling pathways is initiated typically by the activation of $G\alpha_s$ protein. Following receptor ligand binding, the α subunit of the Gs protein activates adenylyl cyclase, which triggers the generation of cAMP from ATP (Fig.1-4) (Hanoune *et al.*, 2001). In addition, adenylyl cyclase activity can be inhibited by ligands that activate $G\alpha_i$ and/or cAMP can be degraded

by phosphodiesterase. Thus, the intracellular levels of cAMP are regulated by the balance between the activities of two enzymes, adenylyl cyclase and phosphodiesterase (Maurice *et al.*, 2003; Tasken *et al.*, 2004).

Intracellular cAMP regulates various cellular functions as primarily through two downstream effectors, PKA (protein kinase A), the classic effector of cAMP (Skalhegg *et al.,* 2000), and the alternative cAMP effectors Epac (Exchange protein directly activated by cAMP).

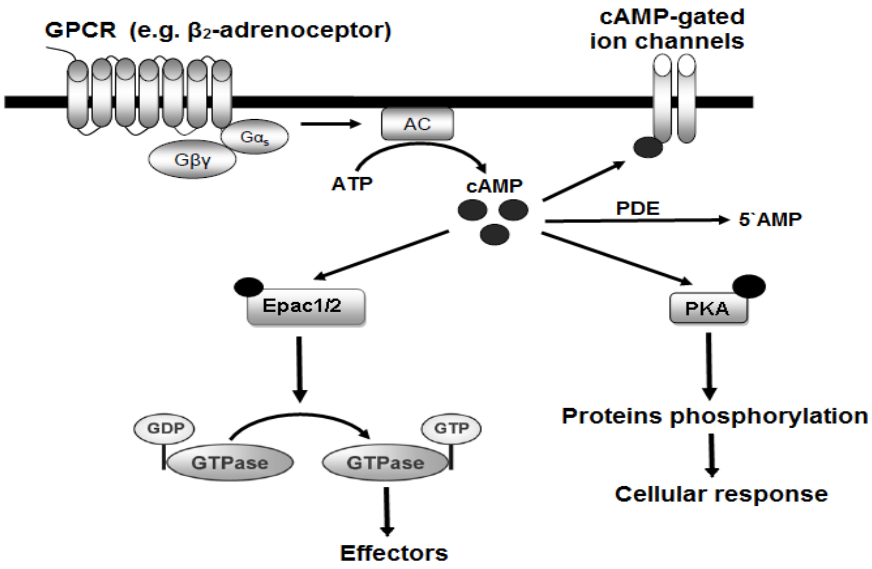


Figure 1-4: Schematic diagram of cAMP-mediated signaling pathways in the cells. AC, adenylyl cyclase; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; 5'AMP, 5'-adenosine monophosphate; Epac1/2, exchange protein directly activated by cAMP; GDP, guanosine diphosphate; GTP, guanosine triphosphate; PDE, phosphodiesterase, PKA, protein kinase A.

1.4.1.1. Protein kinase A (PKA)

PKA, the best characterized cAMP effector, is a serine/threonine kinase that in its inactive state exists as a heterotetramer holenzyme of two regulatory and two catalytic subunits. Three different genes encode for the catalytic subunits (Cα, Cβ, and Cγ) and four different genes for the regulatory subunits (RIα, RIβ, RIIα, and RIIβ), that are differentially expressed in various tissues and display different subcellular localization. Two types of the heterotetrameric PKA holoenzyme have been reported, type I ($RI\alpha_2C_2$, $RI\beta_2C_2$) which is predominantly found in cytoplasmic, and type II ($RII\alpha_2C_2$, $RII\beta_2C_2$) which is mainly associated with specific subcellular structures and compartments (Taylor *et al.*, 2004; Pidoux *et al.*, 2010). The variations

in tissue distribution and the composition of PKA holoenzymes may be related to the variety of biological functions that are mediated by PKA (Skalhegg *et al.*, 2000).
Activation of PKA occurs upon elevation of cAMP levels. Accordingly, the cooperative binding of two cAMP molecules to each of the regulatory subunits induces conformational changes that lead to dissociation of the holoenzyme and the release of catalytic subunits, which in turn become enzymatically active. The active catalytic subunits can then affect a variety of different cellular activities by phosphorylating a wide range of cytoplasmic and nuclear enzymes on serine and/or threonine residues, thereby playing an essential role in the regulation of various cellular functions such as gene regulation, cell cycle, proliferation, differentiation, collagen synthesis, cell migration and metabolism (Tasken *et al.*, 2004; Cheng *et al.*, 2008)

1.4.1.2. Exchange proteins activated by cAMP (Epac)

Epac, also known as cAMP- regulated guanine-exchange factors (cAMP-GEF), is another important intracellular cAMP effector. Epac is a guanine nucleotide exchange factor (GEF) for the Ras family of small GTPases, Rap (Bos, 2006). Rap cycles between an inactive GDP-bound form and an active GTP-bound form. Two isoforms of Epac (Epac1 and Epac2) which share similar structures have been identified (de Rooij et al., 1998; Kawasaki et al., 1998). They are encoded by two distinct genes. Epac1 is ubiquitously expressed, whereas Epac2 is mainly expressed in the brain and endocrine tissue (Kawasaki *et al.*, 1998). Epac proteins are multi-domain proteins containing a catalytic subunit at the C-terminus and a regulatory subunit at the N-terminus, whereas Epac2 has an additional N-terminal cyclic-nucleotide-binding (CNB) domain, which is not required for the regulation of Epac 2 in vitro (Bos, 2006). The regulatory subunit consist of a Dishevelled-Egl-10-Pleckstrin (DEP) domain (Fig.1-5), which is responsible for membrane localization, and a cAMP-binding domains (CBD) homologous to the CBD of PKA, that is needed for activation by cAMP (de Rooij *et al.*, 1998). The catalytic subunit, which possesses GEF activity, is composed of (i) a Ras exchange motif (REM) which functions as bridge between the regulatory and the catalytic region to stabilize the CDC25 homology domain, (ii) a Ras association domain (RA) (Fig.1-5) which allows particularly Epac2 interaction with Ras (Li *et al.*, 2006), and (iii) a CDC25 homology domain (CDC25HD) responsible for the guanine nucleotide exchange function of

Epac-proteins (Li *et al.*, 2006). Crystallographic studies demonstrated that, in the absence of cAMP, the CNB domains sterically hinder Rap binding to the catalytic site, thereby maintaining Epac proteins in an auto-inhibited state (Rehmann *et al.*, 2003; Rehmann *et al.*, 2006). The binding of cAMP to Epac relieves the catalytic domain from the inhibitory constraints to enable interactions with their effectors (Rehmann *et al.*, 2003; Rehmann *et al.*, 2006).

Epac proteins (Epac1 and Epac2) bind cAMP with affinities similar to that of PKA, suggesting that Epac and PKA respond to similar cAMP concentrations (Dao *et al.*, 2006). Furthermore, Epac and PKA can act independently (Huang *et al.*, 2008; Haag *et al.*, 2008b), synergistically (Hochbaum *et al.*, 2008; Petersen *et al.*, 2008) or antagonistically (Mei *et al.*, 2002; Huston *et al.*, 2008) in regulating specific cellular functions on downstream signalling targets, depending upon specific cellular contexts and their relative abundance, distribution, and localization.

Epac proteins are important for cell functioning. Hence, it is well known that they are implicated in several cAMP-mediated cellular functions, such as gene expression, cell differentiation, cell adhesion, secretion/exocytosis, cell-cell junction and apoptosis (Bos, 2006; Cheng *et al.*, 2008; Roscioni *et al.*, 2008).

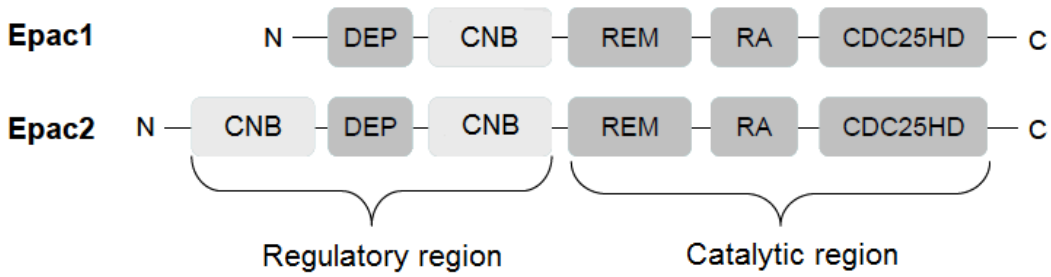


Figure 1-5: Schematic diagram of multi-domain proteins of Epac1 and Epac2. CNB, cAMP-binding domain; DEP, Desheveled-Egl-10-Pleckstrin; CDC25-HD, CDC25-homology domain; REM, Ras exchange motif; RA, Ras-association domain. Adapted from (Bos, 2006).

Cell-permeable cAMP analogs, such as 8-pCPT-2'-*O*-Me-cAMP and 6-Bnz-cAMP which selectively activate Epac or PKA, respectively, have been identified. These analogs are generally used as specific pharmacological tools to study and distinguish between Epac- and PKA-mediated signalling pathways (Enserink *et al.*, 2002; Christensen *et al.*, 2003; Holz *et al.*, 2008). In addition, as Epac proteins are insensitive to PKA inhibitors, PKA inhibitors such as Rp-8-CPT-cAMPS might be

used to further verify PKA- and Epac-dependent cAMP signalling (Holz *et al.*, 2008). Specific pharmacological inhibitors of Epac proteins are not available.

1.4.2. Muscarinic receptors

Five subtypes of muscarinic acetylcholine receptors (M_1-M_5) have been described thus far, which are encoded by five different genes located on chromosomes 11, 7, 1, 11 and 15, respectively (Caulfield *et al.*, 1998). They are members of the superfamily of hormones and neurotransmitter receptors. Muscarinic acetylcholine receptors belong to the GPCRs Rhodopsin family (Wess, 1996), which regulate the activities of both ion channels and enzymes involved in the regulation of intracellular second messenger pathways. Muscarinic receptors are widely expressed in various tissue, and are involved in many functional processes, including the regulation of smooth muscle contraction, cellular proliferation and mucus secretion. M_1, M_2, and M_3 receptors represented the main muscarinic receptors in airways and lung tissue of most mammals including human (van Koppen *et al.*, 1987). M_1 receptors are mainly expressed in the peripheral lung tissues and the alveolar walls (Roffel *et al.*, 1987; Mak *et al.*, 1992). The M_2 receptor is located mainly on the cell membrane of smooth muscle cells, where it acts as functional antagonism to β_2-adrenoceptor-induced airway smooth muscle-mediated relaxation, and as presynaptic inhibitory autoreceptor on parasympathetic nerve endings (Zaagsma *et al.*, 1997; Belmonte, 2005). M_3 receptors are the predominant muscarinic receptors on submucosal glands and thus significantly involved in the pathophysiological mucus formation (Barnes, 2004). In human lung fibroblasts, M_1-M_4 have been detected, but the M_2 receptor represented the major subtype (Matthiesen *et al.*, 2006). Activation of M_2 receptors in human lung fibroblasts, enhanced proliferation (Matthiesen *et al.*, 2006; Matthiesen *et al.*, 2007) and collagen synthesis (Haag *et al.*, 2008a) through activation of the ERK1/-2 MAPK pathway. In addition, muscarinic receptors are also involved in the human lung fibroblasts transition into myofibroblasts, through increase in ERK1/-2 MAPK phosphorylation, RhoA activation, cAMP inhibition and autocrine TGF-β release (Milara *et al.*, 2012).
M_1, M_3 and M_5 receptors couple predominantly to $G\alpha_q$ proteins (PTX-insensitive) to activate PLC, and thereby inducing formation of IP3 and DAG, resulting in increase in intracellular calcium release and activation of PKC, respectively (Wess, 1996). M_2 and M_4 receptors preferentially couple to the inhibitory G protein ($G_{i/o}$) (PTX-

sensitive), inducing inhibition of adenylyl cyclase, thus reduce cAMP production. However, the M_2 receptors can also couple to $G\alpha_s$ and $G\alpha_{q/11}$ family, resulting in stimulation of cAMP synthesis and phosphatidylinositol accumulation, respectively (Michal *et al.*, 2007).

1.5. Mitogen activated protein kinases (MAPKs)

MAPKs are serine/threonine kinases that play key roles in signal transduction from the cell surface to the nucleus. MAPKs regulate a diverse range of cellular processes, including growth, proliferation, differentiation and apoptosis and are implicate in a number of disease states including chronic inflammation and cancer. In mammalian cells, five different families of MAPKs have been identified, the extracellular signal-regulated kinases (ERK1/-2), c-Jun N-terminal kinases (JNK1,2 and 3); p38 kinase isoforms (α, β, γ and δ), ERK3/-4 and the ERK5 (Roux *et al.*, 2004). Among these families, ERK1/-2, JNKs and p38 kinases are implicated in human diseases and are targets for drug development (English *et al.*, 2002; Manning *et al.*, 2003).

MAPK signalling pathways can be activated by a vast array of extracellular stimuli. However, every MAPK-family usually becomes activated in response to a particular set of stimuli. In general, ERK1/-2 is activated primarily in response to mitogenic stimuli like cytokines, growth factors and hormones. On the other hand, JNK and p38 MAPK cascades, which are also called stress-activated protein kinases (SAPKs), are mainly activated in response to stress stimuli like inflammation and apoptosis (Fig.1-6) (Roux *et al.*, 2004; Qi *et al.*, 2005; Whitmarsh, 2007). Each family of MAPK cascades consist of a core module of three evolutionarily conserved, sequentially acting protein kinases termed, MAPK, MAPK kinase (MAPKK), and MAPKK kinase (MAPKKK). Activation of MAPK is achieved by phosphorylation of both conserved threonine and tyrosine residue within their activation loop, which is catalyzed by dual specificity MAPK kinases (MAPKK or MEK). MEK activity, in turn, is regulated via phosphorylation by a MEK kinase (MAPKKK or MEKK) and/or by G-protein (Roux *et al.*, 2004; Qi *et al.*, 2005). In this study the ERK1/-2 signaling was investigated more closely, therefore the following description will focus on ERK1/-2 as components of MAPKs.

ERK is the best characterized MAPK families and the Raf/MEK/ERK pathway represented one of the best characterized MAPK signalling pathways (Rossomando *et al.*, 1989; Peyssonnaux *et al.*, 2001). The mammalian ERK module also know as classical mitogen kinase cascade consists of the MAPKKKs A-Raf, B-Raf, and Raf-1, the dual specificity kinase MEK, (MAPKK MEK1/-2) and the classical MAPK ERK1/-2. Human ERK1/-2 exhibit 84% sequence homology and are uniquely activated through the sequential phosphorylation of the classical MAPK cascade.

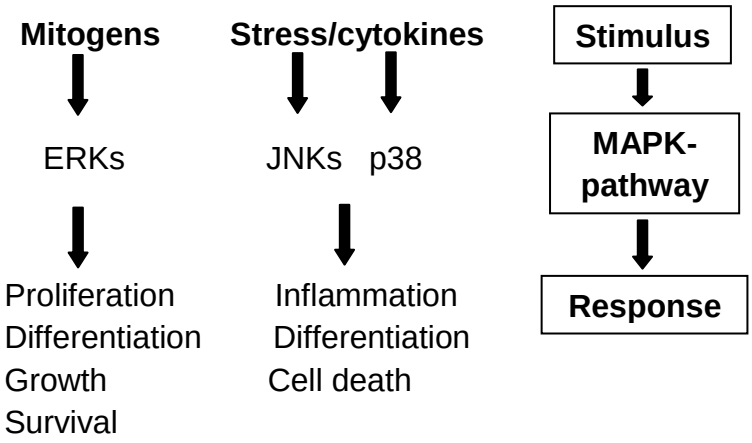


Figure 1-6: Schematic diagram of MAPK signaling pathways in mammalian cells.

1.5.1. Raf/MEK/ERK pathway (MAPK signalling cascade)

The cellular response to mitogens including growth factors, inflammatory cytokines is frequently mediated by GPCR or intrinsic protein receptor tyrosine kinases (RTKs). Raf/MEK/ERK signaling cascade is strongly activated by mitogenic stimuli like growth factors and inflammatory cytokines, often initiated by agonist stimulation of cell surface receptors, like RTKs and GPCR, and mediated through various isoforms of the small GTP binding protein Ras (McKay *et al.*, 2007).

The binding of a ligand to the corresponding RTK induces its oligomerisation and subsequent autophosphorylation in tyrosine residues. The activated RTKs promote Ras activation through recruitment adaptor proteins bearing SH2 and SH3 motifs (Shc and Grbr2, growth factor receptor-bound protein 2) that bind to an exchange factor for Ras, SOS (Son of Sevenless). SOS catalyzes the conversion of inactive Ras–GDP to active Ras–GTP. Ras–GTP promotes the activation of Raf isoforms and recruits them to the plasma membrane. The activated Raf in turn phosphorylates the dual specificity kinases MEK1/-2, which in turn phosphorylate ERK1/-2 within a conserved residue in their activation loop. Amplification through this signaling

cascade is such that activation of solely 5% of Ras molecules is sufficient to induce full activation of ERKs (Hallberg *et al.*, 1994). In quiescent cells, MAPKs are anchored in the cytoplasm due to interaction with a variety of anchoring proteins, including MEK1/-2, however upon activation they dissociate from the cytoplasmic anchoring complex and translocate into the nucleus (Volmat *et al.*, 2001; Shaul *et al.*, 2007). The most important role of MAPKs is the regulation of gene expression. For this action, activated MAPKs translocate to the nucleus and regulate the activity of different transcription factors (Volmat *et al.*, 2001; Shaul *et al.*, 2007).

1.5.2. G-protein coupled receptor signaling to ERK MAPK

It is well established that many of the cellular responses mediated by GPCR do not only involve the stimulation of conventional second-messenger-generating system, but result from the functional integration of a complex network of intracellular signalling pathways. Among these, the MAPK pathways, those mediated by ERK1/-2, JNK and p38 MAPK, are strongly activated by GPCR (Caunt *et al.*, 2006; Goldsmith *et al.*, 2007). One of the main cellular effectors activated by GPCRs is ERK (Rozengurt, 2007). G-protein-dependent ERK activation leads to the translocation of active ERK to the nucleus. The subcellular location of activated ERK1/-2 determines the downstream signaling cascades (Luttrell *et al.*, 2003). Various substrates of ERK1/-2 are found in the nucleus, including nuclear transcription factors that participate in gene transcription, cell proliferation and differentiation. Activation of MAP kinases, particularly of ERK1/-2 is now recognized to be a major signal transduction pathway of many GPCRs.

Most of the mechanisms by which GPCRs activate ERK entail transduction by heterotrimeric G proteins. Gαi and Gαq were the first identify subunits involved in GPCR-mediated activation of MAPK (Gutkind, 2000; Marinissen *et al.*, 2001). Suggested mechanisms for MAPK stimulation are through PLCβ, PKC, second messengers and/or the small G protein (Gutkind, 2000; Marinissen *et al.*, 2001).

Activation of MAPK by GPCR can be mediated through their Gα and/or Gβγ subunits of the G protein (Crespo *et al.*, 1994; Marinissen *et al.*, 1999) depending on the MAPK and the specific stimulus. Because of the abundance of G_i proteins, their βγ subunits are considered to be a main source of the βγ subunit used for transduction in heterologous pathways (Belcheva *et al.*, 2002). The βγ-complex can activate

PLCβ and thereby induce ERK activation (Schwindinger *et al.*, 2001; Rozengurt, 2007).

There are different mechanisms by which GPCRs activate ERK. $G_{\alpha i}$-coupled receptors often act through Gβγ-mediated effects on phosphatidylinositol 3-kinase (PI3K), while $G\alpha_{q/11}$-coupled receptors-directed phosphorylation and activation of ERK involves a pathway that is sensitive to inhibitors of PLC, PKC and/or intracellular Ca^{2+} (Werry *et al.*, 2005).

ET-1-mediated responses, including MAPK activation, are mediated by $G_{\alpha q}$, $G_{11/12,}$ $G_{\alpha i}$, and $G_{\beta Y}$ (Miyauchi *et al.*, 1999; Arai *et al.*, 2003). Activation of ERK1/-2 in response to ET-1 in human VSMCs is predominantly mediated by ET-A receptors through upstream signal molecule, including PKC, PKA and PI3K (Chen *et al.*, 2009). Furthermore, activation of the ERK1/-2 cascade by GPCRs signaling via PTX-sensitive and -insensitive G proteins has been reported for a broad array of ligands, including ET-1 (van Biesen *et al.*, 1996), and ET-1 signaling to MAPK involves βγ-dependent and independent pathways (Miyauchi *et al.,* 1999).

1.6. Rho kinase

Rho kinase is a serine-thronine kinase, the main effector of Rho and the main downstream targets of RhoA, which exists in two isoforms: Rock1 (Rho-associated protein kinase-1) and Rock2 (Rho-associated protein kinase-2) (Fukata *et al.*, 2001). Rho (Ras homologous) protein is a subfamily member of the small GTPases superfamily related to Ras, that act as key regulator of extracellular-stimulus-mediated signaling networks that regulate a variety of fundamental cellular activities, including actin organization, cell cycle progression and transcriptional regulation of gene expression (Etienne-Manneville *et al.*, 2002; Shimokawa *et al.*, 2007). Of the more than twenty identified human Rho GTPases, Rho, Rac1 and Cdc42 are the most intensively characterized family members (Wennerberg *et al.*, 2004). The activation of Rho is regulated by GTPase activating proteins (GAPs), guanine nucleotide exchange factors (GEFs), and guanine nucleotide dissociation inhibitors (GDIs). GAPs enhance the intrinsic GTP hydrolysis rate and thus attenuate Rho protein activity by promoting the formation of the inactive GDP-bound form, whereas GEFs catalyze the exchange of GDP for GTP and thereby activate Rho. GDIs inhibit GDP/GTP exchange and activation of the Rho GTPases (Sah *et al.*, 2000; Satoh *et*

al., 2011). Furthermore, the majority of Rho protein members undergo carboxy (C)-terminal post-translational modification by isoprenoid lipids, which is important for facilitating their membrane association and subcellular localization crucial for their biological activity, and which can also play essential roles in protein-protein interactions and protein stability. Rho proteins have a carboxyl-terminal CAAX tetrapeptide motif (C=cysteine, A=aliphatic amino acid, X= terminal amino acid) that is necessary for post-translational modification. The post-translational modification also termed protein prenylation, is initiated by the attachment of an isoprenoid lipid to the conserved cysteine residue of the tetrapeptide motif. Either farnesyl or geranylgeranyl isoprenoid is covalently attached to this cysteine by protein farnesyltransferase or protein geranylgeranyltransferase-I, respectively. Prenylation is followed by a proteolytic cleavage of the terminal AAX residues and finally the prenylated cysteine is methylated.

1.6.1. G-protein coupled receptor signaling to Rho kinase

Activation of GPCRs switches RhoA from an inactive, GDP-bound conformation to an active GTP-bound conformation, which binds to and activates target molecules (Loirand *et al.*, 2006). It is well documented that, RhoA, and in turn Rho kinase can be activated by a large variety of GPCRs-induced signaling (Bhattacharya *et al.*, 2004) particularly downstream of $G_{12/13}$ protein subtypes of the G-protein family through interaction with specific RhoGEFs (Sah *et al.*, 2000). For instance, $G_{12/13}$ can recruit RGS domain-containing GEFs such as PDZRhoGEF, Leukemia-associated Rho GEF (LARG) and p115-RhoGEF (Fukuhara *et al.*, 2000). RhoGEFs may also inactivate these $G_{12/13}$ G-protein by elevating their GTPase activity via regulator of G protein signaling (RGS) domain of p115RhoGEF, a GTPase-activating protein for $G_{12/13}$ (Kozasa *et al.*, 1998; Chen *et al.*, 2005; Hains *et al.*, 2006). In NIH3T3 cells, ET-1 has been shown to mediate Rho activation through the $G_{12/13}$ (Mao *et al.*, 1998). RhoA can also be activated by $G_{q/11}$ coupled receptors, which normally couple to PLC, this is facilitated exclusively by a $G_{q/11}$-selective GEF, p63RhoGEF (Lutz *et al.*, 2005). The majority of GPCR that regulate cellular Rho-dependent responses, including actin cytoskeletal responses, smooth muscle contraction, gene transcription and cell growth can couple to more than a single class of heterotrimeric G-protein. For example lysophosphatidic acid (LPA) and

thrombin mediate cellular responses through both pertussis toxin-sensitive and insensitive G-proteins (Sah *et al.*, 2000).

In cardiomyocyte, ET-1-induced alterations in cell size, protein production, gene transcription and myofibril organization are inhibited by RhoA and Rho-kinase inhibitors such as C3 toxin (bacterial toxin C3 transferase from clostridium botulinum) and Y-27632 respectively (Kuwahara *et al.*, 1999; Charron *et al.*, 2001). The ROCK inhibitor Y-27632 totally blocked ET-1-mediated contraction in the granulation tissue of sprague-dawley rats, suggesting that activation of Rho/ROCK pathway by ET-1 is critical in regulating in vivo myofibroblast contraction (Tomasek *et al.*, 2006). In addition, Rho/ROCK signalling is required for ET-1-induced myofibroblast resistance to apoptosis (Horowitz *et al.*, 2012). In Swiss 3T3 cells, inhibition of RhoA by C3 toxin or by a dominant-negative mutant caused cells to arrest in the G_1 phase of cell cycle (Yamamoto *et al.*, 1993), suggesting an important role for Rho in regulating cell proliferation. Furthermore, activation of Rho by Escherichia coli cytotoxic necrotizing factor-1 reduced cell cycle inhibitory protein $p27^{Kip1}$ and increased DNA synthesis in human smooth muscle cells (Laufs *et al.*, 1999), thus Rho is crucially involved in regulation of cell proliferation.

1.7. Transforming growth factor-β (TGF-β)

TGF-β is the prototypic member of the TGF-β superfamily, an evolutionary conserved family of structurally related dimeric cytokines, which regulate an extensive range of cellular processes, including cell proliferation, cell differentiation, apoptosis, cell migration and cell cycle progression. In addition to this, TGF-β plays also an essential part in embryonal development, tissue repair and immune system modulation (Blobe *et al.*, 2000; Massague *et al.*, 2000b; Attisano *et al.*, 2002). In humans, the TGF-β superfamily consists of at least 33 related polypeptides (Derynck *et al.*, 2008), including TGF-β isoforms, activins, inhibins, growth differentiation factors and bone morphogenetic proteins (BMPs). Members of the TGF-β superfamily are expressed in most cell types (Feng *et al.*, 2005). All TGF-β superfamily members share a cluster of conserved cysteine residues that form a common cysteine knot structure held together by intermolecular disulfide bonds (Heldin *et al.*, 1997; Attisano *et al.*, 1998; Massague, 1998). In humans, three structurally almost identical isoforms of TGF-β (Grande, 1997) encoded by separate

genes have been described, TGF-β1 (Derynck *et al.*, 1985), TGF-β2 (de Martin *et al.*, 1987; Madisen *et al.*, 1988) and TGF-β3 (Derynck *et al.*, 1988). Among these three isoforms, TGF-β1 is the most abundant and ubiquitously expressed isoform in most human tissues and cell types including lung cells (Pelton *et al.*, 1990; Bragg *et al.*, 2001), and is most prominently expressed in many diseases characterised by fibrosis (Border *et al.*, 1992). Therefore TGF-β1 will be the focus of this thesis, referred to as TGF-β.

1.7.1. Synthesis and secretion of TGF-β

All isoform of TGF-β are initially synthesized as a biologically inactive, large precursor proteins (Homodimeric proproteins), which are held together by three intermolecular disulfide bonds. These dimeric proproteins contain of the N-terminal latency-associated peptide (LAP), propepetide and the C-terminal mature TGF-β peptide, dimeric growth factor (Lawrence *et al.*, 1984; Gentry *et al.*, 1988; Gentry *et al.*, 1990), which are protolytically cleaved by furin-like proteases to yield mature growth factor dimer and propeptide dimer, while in the secretory pathway (Fig.1-7) (Dubois *et al.*, 1995). Although cleavage from mature growth factor, the propeptide LAP remains non-covalently associated with the dimeric growth factor and their interaction makes the mature TGF-β inactive. This complex is referred to as latent TGF-β or small latent complex (SLC), which is unable to generate signaling through high-affinity TGF-β receptors (Miyazono *et al.*, 1988). Once the SLC has attached to the ECM proteins, LAP is disulfide bonded to a larger secretory glycoprotein known as latent transforming growth factor binding protein (LTBP), resulting in the formation of large latent complex (LLC) (Saharinen *et al.*, 2000), which subsequently becomes covalently linked to ECM proteins. LTBP facilitates the release of L-TGF-β (Miyazono *et al.*, 1991) and allows cells to deposit L-TGF-β in their ECM, which requires activation before being able to bind to cell surface TGF-β receptors and to exert a target function (Annes *et al.*, 2003; Rifkin, 2005). TGF-β is secreted as either a SLC or LLC. However most cells, including lung fibroblasts, secrete TGF-β into extracellular matrix as a large latent complex (LLC). There are four LTBPs which have varying degree of functional and structural similarities and differences. LTBP-1 and -3 form effectively complexes with all three TGF-β isoforms, whereas LTBP-4 can form complexes only with TGF-β1 (Saharinen *et al.*, 2000). On the contrary, LTBP-2 is unable to form complexes with any of the TGF-βs (Saharinen *et al.*, 2000).

To initiate TGF-β signalling, latent TGF-β requires biological activation by release of the mature TGF-β from latent precursor (LAP). Activation of latent TGF-β is a rate-limiting step in TGF-β bioavailability (Annes *et al.*, 2003). There are several mechanisms by which TGF-β may be activated, including proteolytic activation which occurs through interaction with matrix metalloproteinases (MMP-2 and -9), thrombospondin-1 (TSP-1), plasmin or the integrins β_6 and β_8 (Gibbons *et al.*, 1992; Annes *et al.*, 2003; Leask *et al.*, 2004; Jenkins, 2008). TGF-β can also be activated through physical processes such as acidification, extreme temperature changes, and oxidation (Sullivan *et al.*, 2008). Moreover, synthesis of TGF-β and its release from the inactive latent complex can be increased by variety of molecules such as ET-1 and angiotensin II (Leask *et al.*, 2004) as well as inflammatory mediators like TNF-α (Sullivan *et al.*, 2009). Once released from LAP, TGF-β can act in an autocrine or paracrine manner.

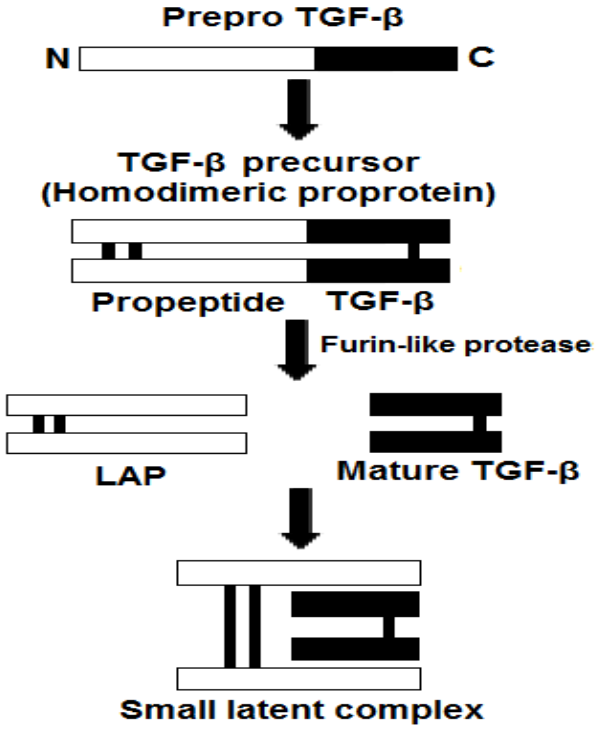


Figure 1-7: Schematic diagram of the biosynthesis of transforming growth factor-β (TGF-β). LAP, latency-associated peptide. Adapted from (ten Dijke *et al.*, 2007).

1.7.2. TGF-β signaling

The TGF-β family members signal through a heteromeric complex of transmembrane type I and type II serine/threonine receptors (Heldin *et al.*, 1997;

Derynck *et al.*, 2003; ten Dijke *et al.*, 2004; Massague *et al.*, 2005). In humans, seven type I receptors (also known as Activin receptor like kinases, ALK1-ALK7) and five type II receptors have been identified, which are generally considered to exist as homodimers (Derynck *et al.*, 2003). The type I receptor is characterized by a highly conserved GS domain containing a repetitive glycine serine motif between the transmembrane and kinase domains (Rosenzweig *et al.*, 1995).

The TGF-β signaling is initiated by binding of the active TGF-β molecule to the TGF-β type II receptor (TβR-II), which induces recruitment of TβR-I forming a heterotetrameric complex (Fig.1-8). The constitutively active TβR-II phosphorylates TβR-I on serine residues within its GS domain causing activation of type I receptors (Fig.1-8) (de Caestecker, 2004). Intracellular signalling of TGF-β is primarily mediated through the Smad pathway although a number of non-Smad signaling pathways have also been demonstrated (Derynck *et al.*, 2003; Shi *et al.*, 2003). Smads are intracellular proteins, which play a fundamental role in the transduction of receptor signals to specific target genes in the nucleus. Eight Smad homologs have been identified in humans (Smad 1-8) (Heldin *et al.*, 1997), constituting three functional classes: the receptor-regulated Smads (R-Smad), comediator Smads (Co-Smad) and inhibitory Smads (I-Smad). The binding of type I receptor to the Smad 2/3 proteins is facilitated by the Smad anchor for receptor activation protein (SARA) (Attisano *et al.*, 2000). R-Smads (Smads1, 2, 3, 5 and 8) are directly phosphorylated and activated by the type I receptor kinases. Phosphorylated R-Smads in turn form heteromeric complexes with the Co-Smad, Smad4. These complexes translocate to the nucleus where they bind to DNA and regulate the transcription of target genes, either alone or in association with other nuclear cofactors (Massague, 2000). The I-Smads (Smad 6 and 7) prevent TGF-β signaling by competing with R-Smads for binding to activated type I receptors or Co-Smad interaction, thereby inhibiting R-Smad phosphorylation (Shi *et al.*, 2003). Among the R-Smads, Smad2 and Smad3 are substrates for TGF-β type I receptors and mediate both TGF-β and activin signals, whereas Smad 1, 5 and 8 are BMP receptors substrates and mediate BMP signals (Attisano *et al.*, 1998). The R-Smads and Co-Smad contain two conserved domains, termed mad homology (MH) domains 1 and 2 (Shi *et al.*, 1997; Shi *et al.*, 1998). With the exception of Smad2, the MH1 domain is responsible for DNA binding, whereas MH2 domain mediates protein-protein interactions with Co-Smads

and with TβRI receptor, and is involved in the transcriptional activation (Lebrin *et al.*, 2005).

The specificity of biological responses is defined by the type I receptor, which targets particular Smad proteins and thereby initiates distinct intracellular signaling cascades (Massague, 1998; Attisano *et al.*, 2002). ALK1, ALK2, ALK3 and ALK6 mediated phosphorylation of Smad1, Smad5 and Smad8, whereas ALK4, ALK5 and ALK7 phosphorylate Smad2 and Smad3. ALK5 is the main TβR-1 that mediates the majority of cellular responses to the three TGF-β isoforms (Massague, 1998; Massague *et al.*, 2000a), meaning that their major pathway go through phosphorylation and dimerization of Smad 2/3. However, in endothelial cells, it has been reported that TGF-β can also signal through ALK1 and subsequent activation of the Smad 1/5/8 signaling cascade (Oh *et al.*, 2000; Goumans *et al.*, 2003)

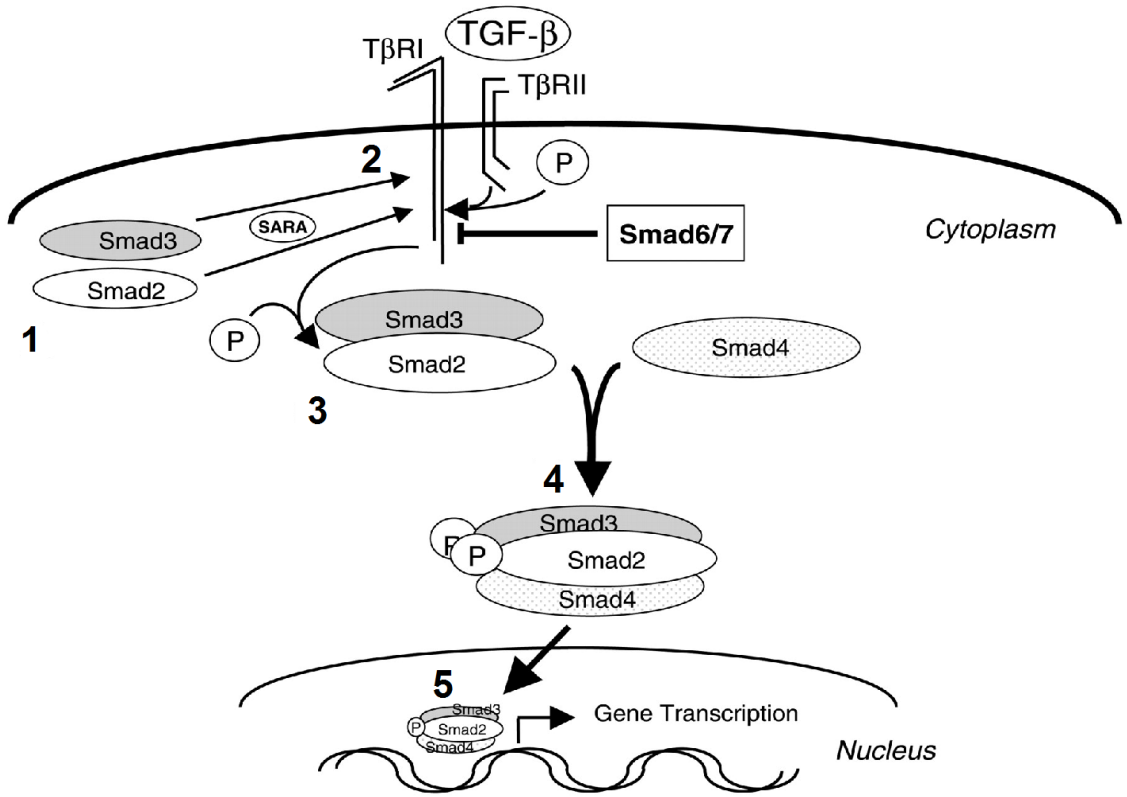


Figure 1-8: Schematic diagram of transforming growth factor–β (TGF-β) signal transduction. R-Smads in the cytoplasm (1) are recruited to the TGF-β receptors (TβRs; 1), where they are phosphorylated (P; 3). A complex of SMAD2 and/or SMAD3 with the common Smad, Smad4, is formed (4), which is subsequently translocated to the nucleus, where it regulates gene transcription (5). SARA, Smad anchor for receptor activation. Taken from (Schnaper *et al.*, 2003).

1.8. Role of ET-1 in lung disease

As mentioned previously, ET-1 appears to be implicated in the pathogenesis of several lung diseases, including pulmonary fibrosis, pulmonary hypertension and fibrotic remodeling processes associated with chronic inflammatory and obstructive airway diseases such as bronchial asthma and COPD.

1.8.1. Pulmonary fibrosis

Pulmonary fibrosis is a recognized feature of various chronic lung diseases, and is central to the pathogenesis of idiopathic pulmonary fibrosis (IPF), a disease of unknown aetiology. It is characterized by excessive proliferation of fibroblasts, excessive deposition of ECM proteins, increase in collagen content of the lung, the presence of distinct accumulations of mesenchymal cells in 'fibroblastic foci' (fibroblasts and myofibroblasts) and alteration of the architecture of the lung resulting in loss of lung function and, eventually, respiratory failure. IPF is thought to be a consequence of abnormal wound healing resulting in progressive scarification (Selman *et al.*, 2001; Thannickal *et al.*, 2004). Various cell types are involved in the pathogenesis of pulmonary fibrosis, including fibroblasts, epithelial cells and smooth muscle cells. Accumulating evidence suggests that ET-1 is a potentially essential contributor in the pathobiology of fibrosing disorders, including those that affect the lung. In vitro data show that ET-1 promotes pulmonary fibroblasts proliferation (Peacock *et al.*, 1992; Shahar *et al.*, 1999; Gallelli *et al.*, 2005) and differentiation into myofibroblasts through the ET-A receptor (Shahar *et al.*, 1999; Shi-Wen *et al.*, 2004) as well as it promotes myofibroblasts resistance to apoptosis (Horowitz *et al.*, 2012). ET-1 also affects production and degradation of matrix by promoting ECM proteins synthesis, inhibiting matrix metalloproteinase-1 expression and promoting matrix remodelling (Xu *et al.*, 1998; Xu *et al.*, 2004; Abraham, 2008). ET-1 displays also strong mitogen activity for endothelial cells and vascular smooth muscle cells (Komuro *et al.*, 1988; Pedram *et al.*, 1997). Additionally, ET-1 has also been described to promote alveolar epithelial cells transition into fibroblast-like cells, fibrotic mesenchymal cells (epithelial mesenchymal transition, EMT) (Jain *et al.*, 2007). Spontaneous increase of ET-1 secretion has been reported by alveolar macrophages recovered from the bronchoalveolar lavage (BAL) of patients with idiopathic pulmonary fibrosis (IPF) (Shahar *et al.*, 1999). The alveolar-associated fibroblasts from patients with IPF show increased expression of α-SMA and

increased ET-1-induced contraction (Fireman *et al.*, 2001). Moreover, patients with idiopathic pulmonary fibrosis have elevated levels of ET-1 in both their plasma and bronchoalveolar lavage fluid (Uguccioni *et al.*, 1995; Reichenberger *et al.*, 2001). The increased levels of circulating ET-1 in patients with lung fibrosis is correlated with the severity of the disease, and is paralleled by an increase in ET-1 synthesis in vivo (Abraham *et al.*, 1997). An increased expression of ECE-1 and co-localised with ET-1 and big ET-1 has also been reported in patient with IPF, and is significantly correlated with disease severity (Saleh *et al.*, 1997). In an experimental model of pulmonary fibrosis induced by bleomycin, levels of ET-1 and ECE are increased (Park *et al.*, 1997; Mutsaers *et al.*, 1998) as well as expression of ET receptors (Wendel *et al.*, 2004). The elevation in the ET-1 level in the lung occurred before the increase in collagen content and was localized within developing fibrotic lesions (Mutsaers *et al.*, 1998). Inhibition of ET receptors with bosentan, a non selective ET receptor antagonist, reduced bleomycin-induced pulmonary fibrosis (Park *et al.*, 1997). Additionally, transgenic mice overexpressing ET-1 spontaneously developed lung fibrosis associated with the accumulation of inflammatory cells, primarily $CD4^+$ cells (Hocher *et al.*, 2000). The overexpression of ET-1 directly in the lungs of mice initiated a fibrogenic response characterized by elevated ECM deposition and myofibroblast formation (Lagares *et al.*, 2012). Numerous cytokines have been implicated in the pathogenesis of lung fibrosis, however TGF-β is the most profibrotic mediator characterized to date and is thought to have a central role in various remodeling diseases, including pulmonary fibrosis (Khalil *et al.*, 1996; Coker *et al.*, 1997; Thannickal *et al.*, 2004). In both normal and fibrotic fibroblasts, TGF-β promotes ET-1 release, which in turn drives production of matrix protein and ECM contraction (Shi-wen *et al.*, 2007). Furthermore, in ET-1 dependent manner, TGF-β induced the expression of profibrotic genes, including type-I collagen, fibronectin and CCN2 and to contract a collagen gel matrix in human lung fibroblasts (Shi-wen *et al.*, 2007). TGF-β and ET-1 can induce resistance to apoptosis in lung fibroblasts through the pro-survival PI3/AKT signalling pathway (Kulasekaran *et al.*, 2009). Additionally, ET-1 enhanced TGF-β synthesis and initiation of EMT was attenuated by a TGF-β-neutralising antibody, demonstrating that ET-1 is an essential mediator of EMT in alveolar epithelial cells, probably acting through ET-A-mediated TGF-β production (Jain *et al.*, 2007).

1.8.2. Bronchial asthma

Asthma is a chronic inflammatory airway disease characterized by bronchial inflammation and airflow limitation that result in recurrent episodes of cough, wheeze and shortness of breath. Various cell types play a role in this chronic disease, in particular, $CD4^+$ T lymphocytes, esinophils and mast cells (Jeffery, 2001), and their mediators, especially TGF-β, IL-4, IL-5, IL-13, IL-17, leukotrienes , platelet-derived growth factor (PDGF) and granulocyte macrophage-colony stimulating factor (GM–CSF) (Busse *et al.*, 2001). Airway limitations in asthma is recurrent and caused by a variety of changes in the airways, these include bronchoconstriction and hyperreactivity with influx of inflammatory cells, mucus production, oedema, and structural alteration in the airways (airway remodling). It is well known that ET-1 has a number of activities that may contribute to each of these processes (Hay, 1999). ET-1 causes immediate bronchoconstriction in asthmatic patients (Redington *et al.*, 1995; Chalmers *et al.*, 1997), it also stimulates mucus secretion, airway oedema, smooth muscle mitogenesis, and bronchial hyperresponsiveness (Hay, 1999). In addition, ET-1 has been reported to increase bronchial reactivity to inhaled antigens (Noguchi *et al.*, 1995) as well as an influx of inflammatory cells (Landgraf *et al.*, 2008). It also enhances cytokine production (Chalmers *et al.*, 1999) and airway remodelling (Glassberg *et al.*, 1994; Finsnes *et al.*, 1998; Goldie, 1999). Airway remodeling is a major feature of asthma (Mauad *et al.*, 2007), and is implicated in the accelerated and progressive loss of lung function observed in asthmatic subjects over time (Lange *et al.*, 1998; Pascual *et al.*, 2005). ET-1 may contribute significantly to the remodeling of the airway reported in asthmatic airways by slowing epithelial cell migration (Dosanjh *et al.*, 2003), increasing production of ECM proteins and differentiation of fibroblasts into myofibroblasts as well as proliferation of airway fibroblasts and smooth muscle cells (Marini *et al.*, 1996; Shahar *et al.*, 1999; Xu *et al.*, 2004).

Elevated ET-1 levels have been detected in BAL (Mattoli *et al.*, 1991; Redington *et al.*, 1995) and bronchial biopsies of asthmatic patients (Redington *et al.*, 1995). The levels of ET-1 in BAL fluid were declined after treatment of acute asthma exacerbations with glucocorticoids and β_2-adrenergic agonists or phosphodiesterase inhibitors (Mattoli *et al.*, 1991; Aoki *et al.*, 1994; Chen *et al.*, 1995). Moreover, ET-1 immunoreactivity was found to be expressed at high levels in bronchial biopsy specimens of patients with asthma (Springall *et al.*, 1991). An increase in ET-1

plasma concentration has also been reported in asthmatic patients and was related to the severity of asthma (Chen *et al.*, 1995). The increase in ET-1 possibly reflects its increased local production from endothelial and/or other cells in the airways of asthmatic subjects. Airway inflammation also leads to increased ET-1 synthesis (Finsnes *et al.*, 1998). Moreover, ET-1 may exert important pro-inflammatory effects in the airways, being both a chemoattractant and an up-regulator of other inflammatory mediators such as the interleukins IL-6 and IL-8, and granulocyte-macrophage colony stimulating factor (GM-CSF) (Mullol *et al.*, 1996). ET-1 also acts as a co-mitogen with a number of growth factors and induces the expression of pro-inflammatory and pro-fibrotic factors including TGF-β (Dubé *et al.*, 2000), TNF-α (Chanez *et al.*, 1996) and fibronectin (Marini *et al.*, 1996).

Pro-inflammatory cytokines, such as TGF-β (Shi-Wen *et al.*, 2006), interleukin (IL)-1 (Yoshizumi *et al.*, 1990) and TNF-α (Chanez *et al.*, 1996) increase the expression of ET-1. There are several lines of evidence that these cytokines are implicated in the inflammatory response of asthma. Levels of TGF-β, TNF-α and IL-1 are elevated in BAL fluid from symptomatic subjects with asthma (Broide *et al.*, 1992; Redington *et al.*, 1997; Chaudhari *et al.*, 2006). An increased expression of TGF-β and TNF-α has been reported in bronchial biopsies from asthmatic subjects with asthma compared with normal subjects (Bradding *et al.*, 1994; Vignola *et al.*, 1997). Accordingly, exposure of bronchial epithelial and/or other cells in the airways to these cytokines may account for the increased production and release of ET-1. It has been shown that the selective ET-A antagonist BQ-123 inhibited immediate and late phase asthmatic responses in guinea pigs (Uchida *et al.*, 1996), and blocking of both ET receptors (ET-A and ET-B) additionally caused a reduction of airway inflammatory response in antigen-challenged animals (Finsnes *et al.*, 1997; Fujitani *et al.*, 1997), suggesting an essential pathophysiologic role for ET in the development of lung inflammation in asthma and highlighting the potential of ET antagonists for the treatment of this disease.

1.8.3. Chronic obstructive pulmonary disease (COPD)

COPD is a common multifactorial disease with a heterogeneous pathogenesis, and is defined according to the recently updated GOLD guidelines as a chronic lung disease characterized by airflow limitation that is not fully reversible.

The airflow limitation is usually progressive and associated with an abnormal inflammatory response in the airways and the lungs to noxious particles and gases.
The characteristic symptoms of COPD are breathlessness, chronic cough, wheezing and excessive mucus production. The disease is characterized by an accelerated decline in lung function, progressive impairment of physical performance and an increased quality of life impairment (Godtfredsen *et al.*, 2008).
Pathologic changes feature of COPD are found at different levels and compartments in the lungs of COPD patients: peripheral airways, proximal airways, lung parenchyma and pulmonary vasculature (Barnes, 2000b; Saetta *et al.*, 2001). These changes are present to differently extend in the affected individual. Cigarette smoking is by far the most important environmental risk factor for developing COPD, however there are many other risk factors including, gene/environment interactions, respiratory infections, occupational exposure and lung growth that may contribute separately or in combination to the development of COPD (Snider, 1989; Barnes, 2000a; Yoshida *et al.*, 2007). COPD is the greatest risk factor for lung cancer among smokers and is found in 50–90% of patients with lung cancer (Young *et al.*, 2009).
The cellular inflammatory response in COPD is characterized by an increase in macrophages, neutrophils and $CD8^+$ lymphocytes in different parts of the lung (Di Stefano *et al.*, 1998; Pesci *et al.*, 1998; Saetta *et al.*, 2001; Hogg *et al.*, 2004). In addition, elevated bronchial deposition of ECM components including collagens and fibronectin have also been reported in patient with COPD (Kranenburg *et al.*, 2006) and may contribute to the decline in lung function and airway remodelling. The inflammatory mediators involved in COPD are less well defined than those in asthma, however significantly elevated levels of leukotriene B4 (chemotactic for neutrophils, LTB4), IL-6, IL-8, TNF-α and TGF-β have been reported in the sputum of patients with COPD (Keatings *et al.*, 1996; Crooks *et al.*, 2000; Bergeron *et al.*, 2006; Perera *et al.*, 2007).
Several studies demonstrated that ET-1 plays an important role in the pathophysiological features of COPD including, mucus secretion stimulation, airway oedema, smooth muscle mitogenesis, and bronchial hyperresponsiviness, as well as ET-1 has also essential proinflammatory effects in the airways (Mullol *et al.*, 1996; Hay, 1999). Sputum and bronchoalveolar lavage ET-1 levels significantly increase during exacerbation of COPD (Roland *et al.*, 2001; Bacakoglu *et al.*, 2003) and this increase in sputum ET-1 levels correlated with the elevation in plasma ET-1 levels

and sputum IL-6 levels (Roland *et al.*, 2001; Trakada *et al.*, 2001; Spiropoulos *et al.*, 2003).

1.8.4. Pulmonary hypertension

Pulmonary hypertension (PH) is a progressive and fatal disease, characterized by vasoconstriction, pulmonary vascular proliferation and remodeling associated with raised pulmonary arterial pressure leading to right ventricular failure (Humbert *et al.*, 2004; Pietra *et al.*, 2004; McLaughlin *et al.*, 2009).

ET-1 serum level is elevated in patients with pulmonary hypertension, and associated with the disease severity and prognosis (Allen *et al.*, 1993; Rubens *et al.*, 2001). In addition, ET-1 clearance in the pulmonary vasculature is decreased in PAH (Stewart *et al.*, 1991). This phenomenon may represent increased ET-1 production and/or reduced clearance by the lung. Furthermore, pulmonary vascular remodeling caused by hyperproliferation of fibroblasts and smooth muscle cells as well as endothelial cells within the pulmonary arterial wall is considered to be one of numerous essential pathophysiologic mechanisms involved in PAH (Humbert *et al.*, 2004; McLaughlin *et al.*, 2009). These cellular processes are associated with over-expression of the vasoconstrictor ET-1 along with a reduction in the vasodilators such as nitric oxide and prostacyclin (Rubin *et al.*, 2002; Budhiraja *et al.*, 2004; Humbert *et al.*, 2004; Racké *et al.*, 2014). Accordingly, and as mentioned previously the potential role of ET receptors in these cellular events, ET receptor antagonists may represent a major advance in the treatment of this disease.

1.9. Aims of the study

ET-1, in addition to its role as vasoconstrictor, also appears to contribute to inflammation and fibrosis during various pathological processes. There is accumulating evidence that ET is involved in the pathogenesis of several lung diseases like pulmonary hypertension, lung fibrosis and fibrotic remodeling processes associated with chronic inflammatory and obstructive airway diseases such as bronchial asthma and COPD.

The lung fibroblasts are connective tissue cells that play an essential role in remodling processes, as a long-term consequence of chronic respiratory diseases. They are the main cells responsible for the synthesis, deposition and remodeling of ECM. Through an increase of proliferation, migration into connective tissues and

synthesis of the ECM components, pulmonary fibroblasts significantly participate in airway remodling processes. Hyper-activation of pulmonary fibroblasts as a result of inadequate control of inflammation, which occurs in chronic respiratory diseases, leads to excessive connective tissue formation, and thereby could contribute to airway fibrosis. The mechanisms underlying the pathophysiology of fibrosis are poorly understood and there are currently no satisfactory therapeutic options available to reverse or prevent excessive fibrosis.

Although there are several reports describing pro-fibrotic effects of ET-1 in rat and human pulmonary fibroblasts, a detailed analysis of the expression of ET isoforms and ET receptors and their potential regulation in human pulmonary fibroblasts as well as a detailed pharmacological characterization of their functions are still missing. Therefore, in the present study, human lung fibroblasts were used as *in vitro* cell model to study whether and to what extent the increased activity of the endothelinergic system might be involved in structural remodelling processes in the context of chronic inflammatory and obstructive airway diseases, including cellular proliferation, differentiation and collagen synthesis, and whether the use of ET receptor antagonists could have beneficial effects on these processes. For this purpose, the following approaches were performed:

1- To determinate the expression pattern and release of ET isoforms and the expression of ET receptors in human lung fibroblasts.
2- To investigate the molecular mechanisms regulating expression and release of ET-1 and its functional significance.
3- To explore the potential effects of TGF-β on the expression and release of ET isoforms and the expression of ET receptors in human lung fibroblasts.
4- To investigate whether the expression of ET-1 in human lung fibroblasts is regulated by muscarinic and/or β-adrenergic mechanisms, and if so, to elucidate the intracellular signaling pathways entailed. In addition, possible functional consequences of an altered ET-1 expression were explored.
5- To investigate whether human lung fibroblasts proliferation and differentiation into myfibroblasts are regulated by endothelinergic mechanisms and if so, to elucidate the intracellular signaling pathways entailed.

6- To investigate whether endothelinergic mechanisms are involved in the regulation of collagen synthesis in human lung fibroblasts and if so, which intracellular signaling pathways are involved.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

Acetic acid	Merck, Germany
Boric acid	Roth, Germany
Bovines serum albumin (BSA)	Bio-Rad, Germany
Calcium chloride	Merck, Germany
Chemiluminescence blotting substrate (POD)	Bio-Rad, Germany
Developing solution (Entwicklerkonzentrat T-Matic)	Adefo-Chemie GmbH, Germany
Dimethylsulfoxid (DMSO)	Merck, Germany
Disodium hydrogen orthophosphate (Na_2HPO_4)	Merck, Germany
Dried milk powder, fat free	Heirler, Germany
Ethanol, absolut	Merck, Germany
Ethylene diamine tetraacetic acid (EDTA)	Sigma-Aldrich
Fixing solution (Fixierkonzentrat T-Matic)	Adefo-Chemie GmbH, Germany
Fetal Calf Serum (FCS)	Biochrom, Germany
Glycin	Roth, Germany
Hydrochloric acid 32% (conc. HCl)	Merck, Germany
Kaleidoscope Marker	Sigma-Aldrich, Germany
Leupeptin	Sigma-Aldrich, Germany
Lumasafe plus scintillation liquid	Lumac LSC,Netherland
Magnesium chloride hexahydrate ($MgCl_2$ x 6 H_2O)	Merck, Germany
Non Essential Amino Acids (100 x)	PAA, Germany
β-Mercaptoethanol	Sigma-Aldrich, Germany
Methanol	Roth, Germany
MOPS SDS running-buffer (20x)	Invitrogen, Germany
Nonidet P-40	Sigma-Aldrich, Germany
Penicillin/Streptomycin solution (10000 U/ml, 10 mg/ml)	Sigma-Aldrich, Germany
Pepstatin A	Sigma-Aldrich, Germany

Phenylmethylsulphonylfluoride (PMSF)	Sigma-Aldrich, Germany
phosphoric acid 85% (H3PO4)	Roth, Germany
Ponceau S	Sigma-Aldrich
Potassium chloride (KCl)	Merck, Germany
Potassium dihydrogenphosphate (KH_2PO_4)	Merck, Germany
Roti-Load 1	Roth, Germany
Sodium chloride (NaCl)	Roth, Germany
Sodium deoxycholate	Sigma-Aldrich, Germany
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich, Germany
Sodium fluoride (NaF)	Merck, Germany
Sodium hydroxide pellets (NaOH)	Merck, Germany
Sodium orthovanadate (Na_3VO_4)	Sigma-Aldrich, Germany
Sodium pyrovate solution (SPS) 100mM	PAA, Germany
Sulfanilamide	Merck, Germany
Sulfuric acid 95% (conc. H2SO4)	Merck, Germany
Trichloro-acetic acid crystals (TCA)	Merck, Germany
Tris-(hydroxymethyl)-aminomethane (Tris)	Roth, Germany
Tris-(hydroxymethyl)-aminomethane-hydrochloride (Tris HCl)	Boehringer Ingelheim, Germany
Triton X-100	Boehringer Ingelheim, Germany
Trypsin-EDTA solution (10x)	Sigma-Aldrich, Germany
Trypthan blue stain (0.4 %)	Sigma-Aldrich, Germany
Tween 20	Sigma-Aldrich, Germany

2.1.2. Radio-active chemicals

[^{3}H]-Proline (specific activity = 1.813 TBq/mmol)	Perkin Elmer, USA
[^{3}H]-Thymidine (specific activity = 37 MBq/ml)	Perkin Elmer, USA

2.1.3. Markers and Nucleotides

Kaleidoscope Marker proteins	Bio-Rad, Germany
Oligonucleotide (dT)18 primer	Eurofins MWG Operon, Germany

2.1.4. Enzymes

DNase (RNase free DNase Set)	Qiagen, Germany
Omniscript reverse transcriptase	Qiagen, Germany
RNase Inhibitor RNasin Plus (40 U/µl)	Fermentas, Germany

2.1.5. Kits for molecular biology

BM Chemiluminescence Blotting Substrate (POD)	Roche, Germany
DC (Detergent compatible) Protein assay kit	Bio-Rad, Germany
ELISA kits	Stressgen, Enzo Life Sciences, Germany
RNeasy Mini Kit	Qiagen, Germany
Omniscript RT Kit	Qiagen, Germany
NucleoSpin® RNA II kit	Macherey-Nagel
QuantiTect® SYBR® Green PCR	Qiagen, Germany
QuickZyme Soluble Collagen Assay kit	QuickZyme BioSciences, Netherlad

2.1.6. Culture medium

Minimal Essential Medium Eagle (MEM) with Earle's Salt and L-glutamine	PAA, Germany

2.1.7. Test substances

Actinomycin (30 µM) — Sigma-Aldrich, Germany
Stock solution prepared in methanol

Big-endothelin (1 nM-100 nM) — Bachem, Germany
Stock solution prepared in sterile water

6-Bnz-cAMP (500 µM) — Biolog life science,Germany
PKA agonist: N^6-benzyladenosine-30,50-phosphate
Stock solution prepared in sterile water

Bosentan (10 µM) — Actelion, Germany
Stock solution prepared in preheated sterile water

BQ-123 (1 µM) Bachem, Switzerland
Selective ET-A antagonist: Cyclo (D-Trp-D-Asp-Pro-D-Val-Leu)
Stock solution prepared in sterile water

BQ-788 (0.1 µM) Bachem, Switzerland
N-*cis*-2,6-dimethylpiperidinocarbonyl-β-tBu-Ala-D-Trp(1-methoxycarbonyl)-D-Nle-OH
Selective ET-B antagonist: Cyclo (D-Trp-D-Asp-Pro-D-Val-Leu)
Stock solution prepared in DMSO

8-CPT-2`-O-Me-cAMP (100 µM) Biolog life science,Germany
Epac agonist: 8-(4-chloropheylthio)-20-O-methylaldenosine-cAMP
Stock solution prepared in sterile water

Cycloheximide (30 µM) Sigma-Aldrich, Germany
Stock solution prepared in DMSO

CGP 20712 (3 µM) Biozol, Germany
1-[2-((3-carbamoyl- 4-hydroxy)phenoxy)ethylamino]-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl)phenoxy]-2-propanol dihydrochloride
Stock solution prepared in sterile water

Enodthelin-1 (0.1-100 nM) Actelion, Germany
Stock solution prepared in sterile water

ICI 118,551 (1 µM) Biozol, Germany
(±)-1-[(2,3-dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(1-methylethyl)amino] 2-butanolhydrochloride
Stock solution prepared in sterile water

Olodaterol (1-10 nM) Boehringer Ingelheim, Germany
Stock solution prepared in sterile water

Oxotremorine M (10 µM) Sigma-Aldrich, Germany
Stock solution prepared in sterile water

PD 98059 (10-30 µM) Sigma-Aldrich, Germany
Stock solution prepared in DMSO

Pertussis toxin (50-100 ng/ml) Sigma-Aldrich, Germany
Stock solution prepared in sterile water

TGF-β human recombinant (0.1-5 ng/ml) Sigma-Aldrich, Germany
Stock solution prepared in sterile 0.1% BSA in 4 mM HCl, further dilutions were done with sterile culture media

Tiotropium bromide monohydrate (100 nM) Boehringer Ingelheim, Germany
Stock solution prepared in sterile water

SIS3 (10 µM) Calbiochem, Germany
Smad3 inhibitor: (6,7-dimethoxy-2-((2*E*)-3-(1-methyl-2-phenyl-1*H*-pyrrolo[2,3-b] pyridine-3-yl-prop-2-enoyl))-1,2,3,4-tetrahydroisoquinoline.
Stock solution prepared in sterile water

Y-27632 (1-10 µM) Tocris, Germany
Rho kinase inhibitor: trans-4-[(1R)-1-aminoethyl]-N-(4-pyridinyl) cyclohexanecarboxamide dihydrocholiride.
Stock solution prepared in sterile water

2.1.8. Antibodies

2.1.8.1. Primary antibody

The following primary antibodies were utilized for Western blot analysis.

Antibody	Origin	Dilution	Supplier
ET-A receptor	Rabbit polyclonal antibody	1:200	Santa Cruz Biotechnology
ET-B receptor	Rabbit polyclonal antibody	1:200	Santa Cruz Biotechnology
α-Smooth muscle actin	Mouse monoclonal antibody	1:1000	Sigma-Aldrich
α-Tubulin	Mouse monoclonal antibody	1:1000	Cedar Lane
p42/44	Rabbit polyclonal antibody	1:1000	Cell Signaling Technology
ERK 2	Rabbit polyclonal antibody	1:500	Santa Cruz Biotechnology

2.1.8.2. Secondary antibody

The following secondary antibodies are conjugated with horseradish peroxydase (HRP) and used for Western blot analysis.

Antibody	Origin	Dilution	Supplier
Anti-mouse Ig	Goat, HRP linked polyclonal antibody	1:5000	Santa Cruz Biotechnology
Anti-rabbit Ig	Goat, HRP linked polyclonal antibody	1:2667	Bio Rad

2.1.9. Equipment

Analytical balance BP221 D, 2258	Sartorius, Netherland
Autoclav 80230	Webeco, Germany
CASY® Cell counter and Analyzer system	Innovatis, Germany
Cell culture incubator, HeraCell 150	Heraeus, Germany
Centrifuges: 54115 C, 5804 R cooled centrifuge Mini-centrifuge MCF 2360	Eppendorf, Germany LMS, USA
Electrophoresis- and blotting chamber: NuPAGE	Invitrogen,Germany
Electrophoresis power supply	Bio Rad, Germany
Film processor, CP100	Agfa, Germany
Fluorescence Microplate Reader Fluorometer	Fluostar OPTIMA, BMG, Germany
Hyperfilm ECL	Amersham, USA
Laminar air flow, HeraSafe	Heraeus, Germany
Liquid scintillation Analyzer, Tri-Carb2100 TR	Packard, Germany
Microscope IMT2-RFL	Olympus, Germany
Mycycler Thermal Cycler System	Bio-Rad, Germany
Neubauer counting chamber	Labomedic, Germany
4-12% Nu PAGE® Bis-Tris Gel	Invitrogen, Germany
PH-Electrode SenTix 81	WTW GmbH, Germany
PH-Meter inoLab 1	WTW GmbH, Germany
PVDF Blotting membrane	Millipore, Germany
Real time PCR machine, MAX3000P Multiplex Quantitative PCR System	Stratagene, USA
Spectophotometer, Smartspec™ plus	Bio Rad, Germany
Therommixer compact	Eppendorf, Germany

2.1.10. Tools for statistical analysis

❖ **GraphPad Software**

Graph Pad Prism 5 software was used for performing biostatistics, graph fittings and scientific graphing. All values are presented as means ± standard error of the mean (SEM) of *n* experiments. The Student's t-test was performed to compare two groups. Statistical significant of differences among more than two groups were evaluated by ANOVA (analysis of variance) followed by Dunnet's or Bonferroni's test. P value of < 0.05 was accepted as statistically significant.

- **MxPro**

  MxPro software was used for evaluation of real-time PCR

- **RFLPscan 2.01**

  RFLPscan software was utilized for semiqauntitatively measured of optical density of bands from Western blot experiments.

2.2. Methods

2.2.1. Cell culture

Most of this work was carried out on MRC-5 human lung fibroblast cell line, but also on some experiments were carried out on primary human lung fibroblasts (phLFb). Both cell types (MRC-5 and phLFb) were grown in Minimum Essential Medium Eagle (MEM with Earle's Salts; PAA) supplemented with 2 mM L-glutamine, non-essential amino acids (NEAA), sodium pyruvate (SPS), penicillin and streptomycin and different amount of FCS (0-15%) depending on the experimental conditions. All cells were cultured in an incubator (Heraus, Heracell 150) with humidified air (95%) and CO2 (5%) at 37°C.

- **MRC-5 human lung fibroblast**

MRC-5 human lung fibroblast cell line was developed in 1966 from normal human lung tissue of an aborted 14 week old male fetus. MRC-5 cells, which grew adherently in culture and exhibit fibroblast morphology, they are capable of 42-46 population doublings before the onset of senescence. American Type Culture Collection number ATCCL-171.

- **Primary human lung fibroblasts (phLFb)**

Cell culture of phLFb were established from histologically normal areas of surgically resected lung tissue, either central or peripheral region of the lung, which was obtained via thoracotomies from lung cancer patients. Tissue was stored until the culture in cold (4°C) 1x PBS. Under the laminar flow, about 4x6 cm piece of lung tissue was sliced into tiny pieces. The resulting tissue pieces were then treated with pronase (1mg/ ml in fibroblast medium with 10% FCS) at 37^{o}C for 30 minutes, thereafter washed twice with 1X BPS and were then placed in cell culture plates, and incubated in human fibroblast medium with 15% FCS. The first medium was

changed after 24 hours of incubation and then twice weekly. After two weeks, fibroblasts had grown out from the tissue, the slices were removed and the cells were allowed to reach confluence. Confluent cells (about 80-90% of confluency) were then passaged by trypsinization further and used for the experiments. The protocol for obtaining human tissue was approved by the local ethics review board for human studies (Ethics Committee, Medical Faculty, University of Bonn, Bonn, Germany), and informed consent was obtained from the patients.

In addition, commercially available primary human lung fibroblasts from healthy Caucasian donors were purchased from PromoCell (Heidelberg, Germany).

2.2.2. Culture of human lung fibroblasts

The frozen cryo-preserved human lung fibroblast culture was thawed rapidly in a water bath at 37°C. Afterward, the cell suspension was transferred to 175 cm^2 culture flask which was pre-incubated in a humidified incubator at 37^oC and 5% CO_2 previously for short time to equilibrate the FCS-containing medium (10% FCS). After 24 hours of incubation the cells were seen to have attached to the flask and, to prevent cell damage, the medium was replaced with 10 ml of fresh 10% FCS culture medium to remove any residue from the cryoprotective agent DMSO. The cells were then allowed to grow and the culture medium was changed twice per week. After the cells reaching confluence, they could be seeded and passaged further.

For cell passage and cultivation, culture medium was aspirated out from the culture flask and the cells were washed with pre-warmed (37°C) sterile 1X BPS. Then, pre-warmed sterile 1X Trypsin/EDTA solution was added to cover the surface of the flask bottom and incubated for approximately 30-45 seconds. Trypsin is a proteolytic enzyme which helps in breaking cell-cell adhesion, thus facilitating the detachment of the adherent cells from the surface of the flask whereas EDTA is a chelator that binds divalent cations such as calcium and magnesium present between the cells allowing trypsin access to the cell-cell and cell-substrate bonds. The tyrpsin was then aspirated out and the detached cells were re-suspended in pre-warm medium containing 10% FCS. Thereafter, the cell suspensions were transferred into a sterile tube and centrifuged for 5 minutes at 1000 RMP. The supernatant was discarded and the cells pellet was resuspended in a known amount of 10% FCS medium. Cell count was carried out in a Neubauer counting chamber using 0.15% trypthan blue.

The counting chamber was filed with mixed solution of 1 part of cell suspension and 4 parts of 0.15% trypthan blue (1:5) and the cells were counted under the microscope. The total number of the cells in whole suspension then can be determined by using chamber factor calculation. Required number of cells were seeded further in a 175 cm^2 culture flask with medium containing 10% FCS or seeded into culture plates to continue cell culture experiments.

To store cells for longer periods of time, cell pellets were resuspended in freezing medium in a ratio of 1:10 (1 part dimetyl sulphoxide, DMSO and 9 parts culture medium with 10% FCS) and transferred to cryogenic vials. The cryogenic vials were frozen to -80^{o}C before being stored in liquid nitrogen (-196°C).

Standard fibroblast culture medium

Basismedium EARLE`S MEM (with L-Glutamine)	
FCS	0-15% (v/v)
Penicillin/Streptomycin	100 U/ml / 100 µg/ml
Non Essential Amino Acids = NEAA	1x
Sodium Pyrovate Solution = SPS	1 mM

Buffers and solutions for cell culture

- **10x PBS (Phosphate buffered saline) pH 7.4-7.5**

KCl	27 mM
KH_2PO_4	15 mM
NaCl	1.38 M
$Na_2HPO4 \times H_2O$	81 mM
Distilled water	

- **1x PBS-Buffer pH 7.4-7.5**

  10x PBS

  1:10 dilution with distilled water

- **1x Trypsin-EDTA-solution**

10x Trypsin-EDTA	1:10
dilution with1x sterile PBS-Buffer	

- **0.15% Trypthan blue staining dye**

0.4% Trypthan Blue Stain	3:8
dilution with 1x PBS-Buffer	

2.2.3. Detection and analysis of RNA and cDNA

2.2.3.1. RNA extraction

Total RNA extraction was performed at room temperature by using either a RNeasy Mini kit provided by Qiagen along with an additional DNase digestion step to avoid any contamination by genomic DNA, or with NucleoSpin® RNA II kit provided by Macherey-Nagel, according to the manufacturer's instructions.

Both procedures (RNeasy and NucleoSpin® RNA II) for RNA purification combine the selective binding characteristics of a silica based membrane with the speed of microspin technology. A specialized high salt buffer system assists the binding of RNA to the silica based membrane.

0.2 - 0.5 Mio fibroblasts were seeded in a 35 mm cell culture dishes or 6 well culture plates, and 2 ml culture medium was added. Depending on the protocol, the cells were incubated in presence or absence of test substances. At the end of incubation period, the medium was aspirated out and the cells were lysed by addition of 350 µl RLT (Qiagen) or 350 µl RA1 (Macherey-Nagel) lysis buffer that was previously treated with β-mercaptoethanol 1:100. The cells lysate was homogenized by centrifugation with the QIA shredder (Qiagen), or with the NucleoSpin filter (Macherey-Nagel), for 2 minutes at speed of 13000 RPM at room temperature and the filtrate was collected in collection tube (2 ml). Ethanol (70%) was then added to the homogenized lysate and mixed thoroughly by pipetting to provide appropriate binding conditions, thereafter the sample was applied to an RNeasy spin column (Qiagen) or NucleoSpin column (Macherey-Nagel), centrifuged at 10000 RPM for 30 seconds. The flow-through was discarded and the spin column was placed on a new collection tube. The wash buffer RW1 (700 µl, Qiagen) or the membrane desalting buffer (350 µl MDB, Macherey-Nagel) was added on the spin column and centrifuged for 30 seconds at 10000 RPM. The flow-through was discarded and the spin column was placed on a new collection tube. In order to remove possible genomic DNA contamination and to enhance PCR quality, an additional DNase treatment step was performed by adding 80µl DNase (10 µl DNase + 70 µl RDD buffer, Qiagen),or 95 µl DNase (10µl DNase + 90 µl reaction buffer for rDNase, Macherey-Nagel) directly onto the spin column and incubating it at room temperature for 15 minutes. After incubation, 500 µl RPE buffer (Qiagen) or 200 µl of RA2 buffer (Macherey-Nagel) was added on the spin column and centrifuged at 10000 RPM for 30 seconds. The

RPE (Qiagen) or RA2 (Macherey-Nagel) buffer wash step was repeated with an extended centrifugation time of 2 minutes. The flow-through was discarded and the spin column was spun dry at 10000 RPM for 30 seconds. The spin column was then transferred onto a new collection tube for the elution, which was done by adding 30-40µl of RNase free water on the spin column and centrifuged at 10000 RPM for 1 minute. To obtain a higher total RNA concentration, the elution step may be repeated. The RNA sample preparation was stored at -80oC.

Each step of the RNA extraction and purification were carried out with RNase free material and solutions to prevent RNA degradation and to get a high quality of RNA preparation.

2.2.3.2. Determination of RNA concentration

The concentration of RNA in the collected samples was determined photometrically by measuring the absorption at wavelenght of 260 nm. In addition, the absorption of blank was measured at wavelenght of 280nm. The ratio of the absorbance at 260 nm to the blank absorbance at 280 nm gives an estimate of the purity of each RNA preparation. The determination was carried out with the SmartSpec^{TM}Plus photometer (BioRad), using quartz cuvettes for measurement. Firstly, the photometer was calibrated with the solvent (RNase-free water) and the samples were then measured in a 1:40 or 1:20 dilution. The RNA concentration was calculated from the absorbance at 260 nm, the dilution factor and a multiplication factor for RNA which is 40. Since 40 µg / ml RNA exhibits an absorbance value of 1 at a wave length of 260 nm.

($A_{260\ nm}$ x 40 x Dilution Factor = RNA concentration in µg/ml)

2.2.3.3. Reverse transcription

Reverse transcription is the process by which complementary DNA (cDNA) is synthesized from an RNA template by means of the enzyme reverse transcriptase. For this purpose, Omniscript Reverse Transcriptase enzyme was used (Qiagen).

For the reaction, an amount equivalent to 1 µg of total RNA was employed as a starting template, making up the volume to 12.5 µl with RNase free water. The RT-Master mix containing 5 mM dNTP-Mix, 1 µM Oligo (dT) 18 primer, 0.25 U RNase inhibitor and 4 U omniscript reverse transcriptase was added to the probe making up volume to 20 µl/probe. The probes were then incubated at 37^{o}C for 1 hr followed by

5 minutes at 93^{o}C, and quickly chilled on ice. Following which the probes were briefly centrifuged and 80 µl of bidest water was then added per probe. The synthesized cDNA were stored at -20^{o}C until further used as the template for the real time PCR reaction.

To prevent RNA degradation care was taken by using RNase free material and all reaction steps were performed on the ice.

RT-Master-Mix

Reagents	**Volume per probe**
10x RT-buffer	2 µl
1 µM Oligo (dT) 18 primer	2 µl
5 mM dNTP-Mix	2 µl
10 U/µl RNase inhibitor (40 U/µl RNase inhibitor RNasin Plus 1:4 dilution with 1x RT-buffer)	0.5 µl
Omniscript reverse transcriptase	1 µl
Total volume	7.5µl

2.2.3.4. Real-time PCR

Real time polymerase chain reaction is a quantitative PCR (qPCR), an in vitro method used to amplify and simultaneously quantify messenger RNA (mRNA). The result obtained by qPCR is sensitive, quantitative, accurate and reliable, wherein data are collected throughout the PCR process as it occurs (in real time), thus combining amplification and detection into a single step.

In principle in qRT-PCR syber green binds to all double-stranded DNA during PCR, causing a fluorescent signal. Determining the quantity of a product is carried out indirectly by the measurement of a fluorescent dye, the signal intensity is proportional to the amount of DNA. The quantification is done in the exponential phase of the PCR, as only in this stage, the PCR efficiency is constant (Pfaffl, 2001). At the exponential phase, the Ct value (Threshold Cycle) is used to calculate the experimental results, at which the fluorescent intensity is significantly above the background fluorescence signal. In addition, because knowing of the PCR efficiency is critical to accurate data interpretation, PCR efficiency was determined for all primers that were used. Under optimal condition, the PCR efficiency (E) reached E = 2 (100%), in which the amount of PCR product doubles during each cycle. The slope

of a standard curve provides an indication of the efficiency of real time PCR. For this purpose, various dilution of known concentration cDNA is measured (1:3 to 1:300) and the Ct values were then plotted against the logarithm of the relative concentration. From the linear regression slope, the PCR efficiency can be determined and this may differ only by 10% from optimal efficiency (slope values correlate to amplification efficiencies between 90% (1.9) and 110% (2.1)). In order to quantify the relative expression of a target gene, an internal standard is required for normalization (Vandesompele *et al.,* 2002), which used to compensate for any difference in concentration between the samples that occur in the analysis of cDNAs. The gene that was used as internal standard, whose expression is independent of cell type, cell stage and external influences (housekeeping genes). For this purpose the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used.

To avoid any contamination all the apparatus used were autoclaved and sterile pipette tips were used. For the real-time PCR, the cDNA was diluted 1:3 with RNase-free water. 5 µl diluted cDNA was used as the template mixed with PCR Master Mix, making up the volume to 15 µl. The master mix containing 7.5 µl QuantiTect SYBR Green PCR Master Mix kit supplied from Qiagen, 0.45 µl sense primer, 0.45 µl antisense primer and 1.6 µl RNase-free water. All samples were run in triplicate for both target genes and housekeeping genes (GAPDH). The real-time PCR was carried out using the Mx3000P Multiplex Quantitative PCR System (Stratagene; 96-well format) using the following specific primer combinations (based on human EMBL sequences):

Primers for real time-PCRs

Primer	**Sequence**
ppET-1 sense	5′-TTATCAGCAGTTAGTGAGAGG-3′
ppET-1 antisense	5′-GAAGGTCTGTCACCAATGTG-3′
ppET-2 sense	5′-TGAGGGACATTTCCACAGTCAAG-3′
ppET-2 antisense	5′-CGGTTGCTCCTGGTTTGTAGC-3′
ppET-3 sense	5′-TATCGGCCTGGTGTCTATAC-3′
ppET-3 antisense	5′-AGTGGACTCCAAGCTAACTC-3′
ET-A receptor sense	5′-GCTCTTTGCTGGTTCCCTGTT-3′
ET-A receptor reverse	5′-GGTCATCAGACTTTTGGACTGG-3′

Primer	Sequence
ET-B receptor sense	5′-TCTTTTGCCTGGTCCTTGTCT-3′
ET-B receptor antisense	5′-GCAGTTTTTGAATCTTTTGCTCAC-3′
Collagen I-α1 sense	5′-TGCTGGTCCCAAAGGTGCTGATG-3′
Collagen I-α1 antisense	5′-GACCAGGCTCACCACGGTCT-3′
GAPDH sense	5′-CTGCACCACCAACTGCTTAGC-3′
GAPDH antisense	5′-GGCATGGACTGTGGTCATGAG-3′

The cycling conditions were 10 min polymerase activation at 95°C (activation of HotStarTaq® DNA Polymerase) and 35-45 cycles at 95°C for 30 sec (Denaturation), 55-58°C (depending on the primers) for 30 sec (Annealing) and 72°C for 30 sec (Elongation). The threshold was automatically set by the software. Fluorescence data from each sample were analysed with the $2^{-[\Delta\Delta Ct]}$ method: fold induction = $2^{-[\Delta\Delta Ct]}$, where $\Delta\Delta Ct$ = [Ct GI (unknown sample) - Ct GAPDH (unknown sample)] - [Ct GI (calibrator sample) - Ct GAPDH (calibrator sample)], GI is the gene of interest. Furthermore, ΔCt = 35 was set as detection limit, and the fold expression over detection limit ($2^{35-\Delta Ct}$) was used as a measure of the expression levels.

2.2.4. Enzyme linked immunosorbent assay (ELISA)

The concentrations of ET-1 in cell culture supernatants were measured by ELISA using commercially available ELISA kits (Stressgen, Enzo Life Sciences GmbH, Lörrach, Germany).

Enzyme linked immunesorbent assay (ELISA) also called enzyme immunoassay (EIA) is a biochemical technique used as analytic and diagnostic tools to detect the presence of antibodies or antigens in a sample. The basic principle of an ELISA is to use an enzyme to detect the binding of antigen (Ag) antibody (Ab). The enzyme converts a colorless substrate (chromogen) to a colored product, indicating the presence of Ag:Ab binding. An ELISA can be used to detect either the presence of Ags or Abs in a sample, depending on how the test is designed.

MRC-5 cells were cultured for 24 h in presence of 10% FCS, followed by 48 h in presence of 1% FCS, and absence or presence of TGF-β. Thereafter, the medium was changed and cells incubated for a further 1 to 8 h. The supernatant were then collected for ET-1 determination by a highly sensitive (detection limit 0.41 $pg \cdot mL^{-1}$)

and specific cross-reactivity for ET-1 100%, ET-2 21%, ET-3 3.6%, human big-ET-1 < 0.1%, using commercially available ELISA kit (Stressgen, Enzo Life Sciences GmbH, Lörrach, Germany). Preparations were performed according to the manufacturer's instructions. The kit contains a polyclonal antibody for human ET-1 and was immobilized on a microtiter plate to bind the ET-1 to the standards or samples. A recombinant human ET-1 protein was used as standard. After a short incubation the sample or standard with ET-1 antibody was washed out and a monoclonal antibody to human ET-1 labeled with the enzyme Horseradish peroxidase was then added. This labeled antibody binds to the human ET-1 captured on the plate. After a short incubation the excess labeled antibody was washed out and MTB substrate solution was added. The substrate reacts with the labeled antibody bound to the human ET-1 captured on the plate. After a short incubation, the enzyme reaction was stopped and the color generated was read at 450nm. The measured optical density was directly proportional to the concentration of human ET-1 in either standards or samples.

Assay procedure

All reagents of the kit were allowed to warm at room temperature for at least 30 minutes prior to opening and all necessary buffers and solutions were prepared according to the manufacturer's instructions. To ensure reproducibility of the results, on each ELISA plate all standard curve dilutions and samples were run in duplicate and results pooled.

For the preparation, wash buffer was prepared by adding 50 mL of wash buffer concentrate to 950 mL of sterile water. And antibody was prepared by diluting 10 µL of the supplied antibody concentrate with 1 mL of antibody diluent for every mL of 1X needed. The diluted antibody must be used within 8 hours. In the meantime, standard curve was prepared in Eppendorf tubes. The fallowing dilutions were made to produce the standard curve to achieve a range of concentrations from 0.78 to 100 pg/ml: eight 1.5 Eppendorf tubes were labled #1 through #8. 450 µL of the assay buffer was then piptted into tube # 1, and 250 µL into tubes #2 through #8. Thereafter, 50 µL of the 1.000 pg/mL standard stock was added into tube number1 and vortexed thoroughly. 250 µL of tube #1 to tube #2 was added and vortexed thoroughly, and this was continued for tubes #3 through #8. Diluted standards should be used within 30 minutes of preparation. The concentration of ET-1 in tubes #1

through #8 will be 100, 50, 25,12.5, 6.25, 3.13,1.56 and 0.78 pg/ml respectively.
For the handling of the sample, an equal volume of 20% acetic acid (AA) was added to the sample and centrifuged at 3000 x g for 10 minutes at 4°C to clarify. The pellet was discarded and the supernatant transferred into a new tube. 200 mg C18 Sep-Pak column equilibrated with one column reservoir volume (CV) 100% methanol (MeOH), followed by one CV water and one CV 10% MeOH. The supernatant was applied to the Sep-Pak column and washed with one CV 10% AA and the washes were then discarded. Thereafter, the column was washed with CVs ethyl acetate. Finally, the sample was eluted slowly by applying 3 mL 100% MeOH/ 0.05 M ammonium bicarbonate (80/20 v/v) and the eluant was collected in a new tube. If samples cannot be assayed immediately, store at -20°C. For the measurement, the eluant was reconstituted with at least 250 µL of the assay buffer and measured immediately. Before beginning the procedure of the assay, the microplate was washed twice by adding 400 µL of wash solution to each well. Thereafter, 100 µL of Assay Buffer was pipetted into the S0 (0 ng/mL Standard) well followed by 100 µL of Standards #1 through #8 into the appropriate wells. 100 µL of the samples was then added into the appropriate wells, the plate is sealed and incubated at room temperature for 1 hour. At the end of incubation, the content of the wells was emptied and washed for total of five times by adding 400 µL of wash solution to every well. After the final wash, to remove any remaining wash buffer, the plate was firmly tapped on a lint free paper towel. Thereafter, 100 µL of the diluted antibody was pipetted into each well, except the blank, and the plate is sealed and incubated at room temperature. The plate was washed again for total of 5 times as described above and 100 µL of the substrate solution (a solution of 3,3',5,5' tetramethylbenzidine (TMB) and hydrogen peroxide) was added to each well followed by incubation at room temperature for 30 minutes. Later, 100 µL of stop solution was added to each well and the plate was read immediately by placing it in a microplate (ELISA) reader. After blanking the plate reader against the substrate blank, absorbance of each well was read at a wavelength of 450 nm using FluoStar^{TM} OPTIMA photometer. The concentration of ET-1 in the tested samples was estimated using the standard curve obtained from the assay procedure.

2.2.5. Determination of cell proliferation and viability

2.2.5.1. [^{3}H]-Thymidine incorporation Assay

Cell proliferation was measured by determining the uptake of [^{3}H]-thymidine into DNA. [^{3}H]-Thymidine incorporation method is the classical and reliable method to measure the newly synthesized DNA (Naito *et al.*, 1987). Cell proliferation assays measure the incorporation of a radiolabeled DNA precursor, [^{3}H]-thymidine, into the replication strands of DNA produced during cell division. Measurement of tritiated thymidine ([^{3}H]-thymidine) incorporation as cells enter S phase is considered to be one of the most familiar and widely used methods for quantifying cell proliferation. Cells incorporate the labeled DNA precursors into newly synthesized DNA, such that the amount of incorporation, measured by liquid scintillation counting, is a relative measure of cellular proliferation.

Human lung fibroblasts cells (MRC-5 or phLFb) were cultured as previously described in section 2.2.2. Cells were then trypsinized, harvested, and seeded into 12-well cell culture plates at a density of 4 x 10^4 or 7.5 x 10^4 or 2 x 10^5 cells per well depending on the protocol being proceeded. Three different sets of protocols were tested to discover under which conditions endothelinergic effects might be particularly prominent on the proliferation rate.

In the first set which was applied in most experiments, cells were seeded into 12-well cell culture plates at a density of 4 x 10^4 cells per well. Cells were initially cultured for 24 hours in the presence of 10% FCS, followed by an additional 18-24 hours under FCS-free conditions. Thereafter, test substances were added and present for 30 hours under FCS-free conditions, and [^{3}H]-thymidine (37 MBq/ml) was present during the last 24 hours (i.e. it was added 6 hours after the addition of test substances).

In a second set of experiments, cells were seeded into 12-well dishes at a density of 5×10^4 cells per well and cultured for 5 hours in the presence of 10% FCS. Subsequently, cells were starved overnight in serum free medium, subsequently test substances were added and present for 30 hours under FCS-free conditions, and [^{3}H]-thymidine (37 MBq/ml) was present during the last 24 hours.

In a third set of experiments, cells were seeded at a density of 2 x 10^5 per well and cultured under FCS-free conditions from the onset in presence of test substances for 30 hours. [^{3}H]-Thymidine (37 MBq/ml) was present during the last 24 hours.

After the end of the incubation period, the culture supernatants were aspirated out and the adherent cells were washed with ice-cold 1X PBS, followed by a denaturation in 5% trichloro-acetic acid (TCA) for 10 minutes at 37^{o}C. Thereafter, TCA was aspirated out and the cells were washed once again with ice-cold 1X PBS and the fixed cells were lysed by incubating with 1 ml 0.1 mol/l NaOH at 37^{o}C for 1 hour, subsequently neutralized with 200 µl Tris HCl (PH 7.4). An equal portions 300 µl of samples were combined with 3 ml of scintillation cocktail, and DNA incorporated radioactivity in the lysates was measured by liquid scintillation spectrometry in a Packard 2100 TR liquid scintillation analyzer. 300 µl of a mixture of 1 ml 0.2 M NaOH with 200 µl 0.2 M HCl in 3 ml of scintillation cocktail was performed as blank reading to correct for counting efficiency. The results of the incorporated [^{3}H]-thymidine into the DNA were expressed either in absolute terms, disintegrations per minute (d.p.m) or as a percentage of the mean value of the controls group of each series of experiments.

Buffers and solutions for proliferation assay

- Trichloro-acetic acid — 5 % (w/v) in bidest H_2O
- NaOH — 0.1 M in bidest H_2O
- 1 M Tris-HCl pH 7.4 — Tris-HCl 1 M
Bidest water
Adjust to PH 7.4 with HCl (conc)
- 1x PBS, pH 7.4-7.5

2.2.5.2. Cell counting

MRC-5 human lung fibroblasts were cultured as previously described in section 2.2.2. Cells were then trypsinized, harvested, and seeded into 12-well cell culture plates at a density of 7.5 x 10^{4} cells per well and cultured under FCS-free conditions from the onset in absence or presence of test substance for 28 or 48 h. At the end of the incubation period cells were trypsinized and resuspended in medium. 200 mL of cell suspension were diluted into 10 mL of a ready-to-use isotonic saline solution (CASYton®). This dilution was counted immediately in a CASY®CellCounter+Analyzer System (Innovatis, Bielefeld, Germany). Cell count is expressed as a percentage of the mean value of the controls of each cell preparation.

2.2.6. Determination of collagen synthesis

2.2.6.1. [^{3}H]-Proline incorporation assay

[^{3}H]-Proline incorporation is a method to detect new collagen synthesis and deposition into the extracellular matrix. The method was originally developed in 1971 by Peterkofsky and Diegelmann and subsequently used in many studies and optimized (Trevisan et al, 1997; Eickelberg et al, 1999; Goulet et al, 2007), and established also in our laboratory (Haag *et al.*, 2008a).

Human lung fibroblast cells (phLFb or MRC-5 cell lines) were cultured as previously described in 2.2.2. Cells were then trypsinized, harvested, and seeded into 12-well dishes at a density of 10^5 per well human lung fibroblasts (phLFb or MRC-5 cell lines) were seeded into 12 or 24 well cell culture plates. Cells were initially cultured for 24 hours in presence of 10% FCS, followed by an additional 18-24 hours under FCS-free conditions. Subsequently, [^{3}H]-proline (37 MBq/ml) was added alone or in combination with the test substances, to the near-confluent fibroblast cultures, since fibroblasts synthesised under confluent conditions considerably more collagen than under sub-confluent conditions (Shen and Strecker, 1975). The Incorporation phase lasted for further 24 hours. After the end of the incubation period the medium was removed and the cells were washed twice with 4°C cold 1X PBS, followed by 1–2 h incubation in 1 mL 20% trichloro-acetic acid (TCA) at 4°C. Denaturated cells were scraped off, transferred into a reaction tube and centrifuged at 13000 RPM for 10 min. The supernatant was removed and discarded and the resulting pellet was washed with 1 ml of 10% TCA and centrifuged again at 13000 RPM for 5 min. After the wash steps, the pellet was solubilised by incubating with 300 µl 0.2M NaOH at room temperature for 1 hour, then neutralized with 300 µl 0.2M HCl. An equal portion 300 µl of samples were combined with 3 ml of scintillation cocktail, and radioactivity was measured by liquid scintillation spectrometry in a Packard 2100 TR liquid scintillation analyzer. 300 µl of a mixture of 150 µl 0.2 M NaOH with 150 µl 0.2 M HCl in 3 ml of scintillation cocktail was performed as blank reading to correct for counting efficiency. [^{3}H]-Proline incorporation was expressed either in absolute term, disintegrations per minute (d.p.m.) or as percentage of the mean value of the controls group of each series of experiments.

Buffers and solutions for proline assay

- Trichloro-acetic acid 10/20 % (w/v) in bidest.H_2O
- NaOH 0.2 M in bidest.H_2O
- HCl 0.2 M in bidest.H_2O
- 1x PBS, pH 7.4-7.5

2.2.6.2. QuickZyme collagen assay

Collagen synthesis was additionally assessed by QuickZyme Soluble Collagen Assay (QuickZyme BioSciences, Netherlands). This method is colorimetric assay, which is based on specific binding of the dye Sirius Red to collagen. Following binding of the dye, the collagen-dye complex precipitates, resulting in a coloured pellet. This colour can be released in an alkaline solution.

Cells were cultured for 24 h in presence of 10% FCS, followed by an additional 24 h under FCS-free conditions in absence or presence of test substances. At the end, culture medium was removed, and cells were washed with 4°C cold PBS followed by overnight incubation in 500 µL 0.5 M acetic acid at 4°C. Denaturated cells were scraped off, and the extract centrifuged at 3000 x *g* for 10 min. The supernatant was analysed according to the manufacturer's instructions.

Standard preparation

All reaction steps were performed on the ice to prevent collagen denaturation, and all samples and standards be assayed in duplicate.

Diluted collagen stock was prepared by adding 120 µl of collagen standard solution (714 µg/ml in 20 mM acetic acid) to 1080 µl dilution buffer, resulting in 1200 µl of the 10-fold diluted collagen stock (71.4 µg/ml). The standard curve was made to achieve a range of concentrations from 0 to 10 µg collagen /well, which was prepared as follows: eight Eppendorf tubes were labled #1 through #8. 350 µL of 10-fold diluted collagen stock (71.4 µg/ml) was piptted into tube # 1, 280 µL tube # 2, 210 µL tube # 3 , 140 µL tube #4, 70 µL tube #5, 35 µL tube #6, and 17.5 µL tube #7, followed by piptted 70 µL of addition buffer into tube # 2, 140 µL tube # 3 , 210 µL tube #4, 280 µL tube #5, 315 µL tube #6, 332.5 µL tube #7 and 350 µL tube #8.

Assay procedure:

140 µL of standard was pipetted into appropriate wells of the assay microplate (V-shaped plate) , 140 µL of dilution buffer into appropriate wells for use as blank, and 140 µL of µl of unknown samples into the appropriate wells followed by 60 µL of dye

solution to each well and then was mixed thoroughly by pipetting up and down. The plate is sealed and incubated for 10 min on ice. At the end of incubation, the plate was centrifuged at 3000 x g for 1 hour at 4°C. Thereafter, 100 µl washing solution was added to each well, the supernant and washing solution (unbound dye) was removed by placing the plate at a 45^{o} angle on a (5 cm thick) stack of paper towels. If the solution doesn't easily flow out of the plate, gently move the plat. 250 µl washing solution was added to each well and this solution was removed by placing the plate at a 45^{o} angle on a stack of paper towels as described above and this washing step was repeated. The plate was placed upside down on a paper towel for at least 5 minutes to remove remainder of wash buffer, followed by addition of 150 µl detection solution to the pellets and mixed thoroughly by pipetting up and down. Finally, 100 µl of the coloured solution is transferred to the reading microplate (flat bottom), and the absorbance of each well was read at wavelength of 540 nm using a photometer FluoStar^{TM} OPTIMA.

2.2.7. Detection and analysis of proteins

2.2.7.1. Total protein preparation from adherent cell culture

For protein expression analysis, MRC-5 fibroblasts were seeded into 55mm culture dishes or into 6 well plates at a density of $1x10^{5}$ per dish, or $1x10^{3}$ per well, respectively, and grown in humidified incubator at 37 ^{o}C and 5% $CO_{2.}$ Cells were first cultured for 24 hours in the presence of 10% FCS, followed by an additional 18–24 hours under FCS-free conditions. Cells were then incubated with or without test substances for the times indicated. In inhibitor studies, cells were pre-incubated with vehicle or inhibitor for 30 min prior to stimulation. Culture dishes or plates were then maintained on ice. The medium was aspirated out and the cells were washed with ice cold 1XPBS. The adherent cells were then lysed with 200 µl of 4^{o}C lysis/protein extraction buffer (radioimmunoprecipitation (RIPA)-buffer) containing protease inhibitor (PI). In the case of protein extracts being immunoblotted to test for activated phospho-protein, phosphatase inhibitors (PhI) were added to the lysis buffer in addition to protease inhibitor (PI), to prevent dephosphorylation of the proteins after extraction. Cells were scraped with a cell scraper and lysates. The contents of each well were transferred to 1.5 ml sterile reaction tubes on ice and then centrifuged at 13000 RPM for 15 minutes at 4°C. The supernatants were placed into new tubes

and any debris was discarded. Samples were either frozen at -20°C or directly determined the protein concentration of the supernatant and subjected to SDS-PAGE.

Buffers for protein detection

- **RIPA Lysis Buffer**

TrisHCl	10 mM pH 7.4
NaCl	150 mM
NP-40	1%
Desoxy-cholic acid-Na	1%
Sodium dodecyl sulphate (SDS)	0.1%

- **PI**

0.1 M EDTA, PH 8	2 Mm
125 µg/ml Pepstatin A	0.7 µg/ml (1 µM)
100 mM PMSF	170 µg/ml (1 mM)
10 µg/ml Leupeptin	0.5 µg/ml (1 µM)

- **PhI**

1.75 mM NaF	35 mM
20 mM Na_3VO_4	1 mM

2.2.7.2. Quantification of protein concentrations

The protein concentration was measured using Lowry protein assay. This method is colorimetric assay, which is based on the combination of biuret reaction and Folin-ciocalteau phenol reagent and is referred to as the lowry assay, in which the proteins react with an alkaline copper tartate solution (biuret reaction) and subsequently the copper-treated proteins reduce Folin reagent giving rise to a characteristic blue colour which can be measured colorimetrically by absorbance at wave length 750 nm. The amount of color produced is proportional to the amount of protein. The reagents are from the DC protein assay kit was supplied by Bio Rad and they have been used according to the manufacturer's instructions.

For the assay, 5 µl of the supernatant previously extracted was mixed with 45 µl of 0.1% Tris-Triton-X 100. Then 100 µl of freshly prepared Working solution A (1% reagent S in reagent A) was added to the probe followed immediately by 800µl Reagent B. Likewise 50 µl bovine serum albumin (BSA) standards in the

concentration of 50, 100, 300, 700, 1100, and 1500 µg/ml in 0.1% Triton X100 were mixed with 100 µl of Working solution A and 800µl of Reagent B was added to each probe. For the blank reading, 5 µl of the lysis buffer was treated in a similar fashion. The probes were subsequently vortexed and incubated at room temperature for 15 min. After the incubation, the absorbance was measured spectrophotometrically at a wave length of 750 nm, and the concentration of protein samples was interpolated from the regression line of the standards.

Solutions for protein detection

- 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

2.2.7.3. SDS-polyacrylamide-gelelectrophoresis (SDS-PAGE)

Protein samples were analysed using SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis), which is the most widely used analytical method for separating proteins according to their size (molecular weight) from a mixed protein sample in an electric field, which was performed under denaturating conditions. SDS is an anionic detergent that denatures secondary and non disulphide linked tertiary structures of the proteins by breaking hydrogen bonds and unfolding protein, it binds to the proteins at a constant ratio of 1.4gm SDS per gram of protein, and applies an evenly distributed negative charge to each protein in proportion to its relative molecular mass enabling migration towards the positive pole when run through a polyacyrlamide gel placed in an electric field.

Protein gel electrophoresis was carried out using Nu PAGE® novex® system for western blot supplied by Invetrogen. This system is based upon a Bis-Tris-HCl buffered gel polyacrylamide gel that operates at neural PH (PH 7), that minimizes

protein modifications. To optimize the separation of small to medium-sized of proteins under denaturing conditions, polyacrylamide gradient gels (4-12% Nu PAGE® Bis-Tris Gel, Invitrogen) were used. Protein samples to be analysed were first mixed with reducing agents 1:4 (4x loading buffer, Roth) and then heated for 10 min at 70°C to further denature the proteins by reducing disulphide bonds, thus overcoming some forms of tertiary protein folding, and breaking up quaternary protein structures. The samples were thereafter centrifuged at 1000 rpm for 30 seconds to allow all the contents of each eppendorf tube to collect at the bottom. An equal amount of protein samples were loaded on gradient 4-12% Nu PAGE® Bis-Tris Gel (Invitrogen). 5 µl of the Kaleidoscope marker proteins (Bio-Rad) were loaded as molecular size standards for determines molecular size of other proteins on the gel. The gel electrophoresis run was performed in 1xNu PAGE® MOPS-SDS running buffer (Invitrogen) at a constant voltage of 200 V at approximately 80-90 mA current for about 90 minutes.

2.2.7.4. Western blot analysis

Western blot analysis, also referred to as immunoblot, is a technique used to detect specific proteins in a complicated mixture of different proteins by polyclonal or monoclonal antibodies that is specific to that particular protein. First, proteins are separated by SDS-PAGE and then electrically transferred onto a polyvinylidine diflurid (PVDF) membrane. Proteins on the membrane can be visualized by immunodetection reagents.

2.2.7.5. Protein blotting

The immunoblot was performed using XCell II™ Blot-Module (Invitrogen). Prior to the completion of the gel run, the PVDF membranes were moistened with methanol for a few seconds and washed with distilled water, then equilibrated in the transfer buffer for about 20 min. After separating the proteins by SDS-PAGE, the SDS gel was washed with distilled H_2O to remove any adhering salts and detergents and then equilibrated with 1x transfer buffer for 5 min. At the end of the equilibration period the gel was placed on a sheet of Whatman paper soaked in a transfer buffer. The PVDF membrane was then placed on the top of the gel and the membrane was covered with another sheet of Whatman paper soaked in a transfer buffer, ensuring

that there were no air bubbles which would prevent the transfer of the proteins. The assembly was then covered with sponges soaked in transfer buffers on both sides. This sandwich was then placed in a *XCell II*^{TM} Blot Module. The blot module was placed inside the XCell Sure Lock and then filled with a 4°C transfer buffer. Water was placed in the XCell Sure Lock around the outside of the blot module. The transfers were carried out at constant current of 250 mA, 100V for 90 min.

2.2.7.6. Immuno-detection of blots

Following the protein transfer onto membranes, the membrane was removed from the blot module and placed protein side up in a flat container on a rocker. Following this, the membrane was stained with 0.2% w/v ponceu S (Sigma) for visualizing the protein bands, and to confirm efficient transfer of protein onto the membrane and the lanes were then marked. Next, the stain was removed by washing with water followed by washing with TBS on a shaking platform for 2-3 minutes until the stain was removed. Afterwards, the unspecific binding sites on the membrane were saturated by 5% blocking solution (dried milk powder in 0.05% TBST) over night at 4^{o}C.

The next step, the membrane was incubated for 90 minutes with an appropriate dilution of the primary antibody in 3% blocking solution at room temperature on the rocker. After antibody incubation, the unbound antibody was washed twice for 10 min with 0.1% TBST and blocking twice for 10 min with 3% blocking solution on a rocker. The membrane then was incubated for 45 minutes with appropriate dilution of a horseradish peroxidase (HRP)-conjugated secondary antibody in 3% blocking solution at room temperature on the rocker. Later, the membrane was washed again with 0.1% TBST for four 15 minutes periods on a platform rocker.

In the meantime, the chemiluminescene detection solution was prepared, using BM chemoluminescence blotting substrate peroxidise (POD) kit (Roch) and kept at room temperature. At the end of the final washing step, TBST solution was decanted and the detection solution was added to the protein side of the membrane for 1 min. Finally, the membrane was wrapped into a plastic foil and fixed in an autoradiography cassette and exposed to an X-ray film in a dark room for 5 sec to 5 min depending on the signal intensity. The film was developed by an automatic photo developer (CP-100, Agfa). The developed films were then scanned and RFLP

scan software was used to perform the densitometric analysis of the blots. The results are expressed as arbitrary units related and normalized to the signal of house keeper.

Buffers and solutions for protein gel electrophoresis and immunoblot

- **Running Buffer**

1x NuPAGE MOPS SDS	5% (v/v) 20x NuPAGE MOPS SDS running buffer
	Distilled Water

- **Transfer Buffer**

Tris	25 mM
Glycine	192 mM
Methanol	20% (v/v)
Distilled water	

- **Ponceau S solution**

Ponceau S	0.2% (w/v)
Tris-HCl	3% (w/v)
Distilled water	

- **Tris Buffered Saline (TBS) solution pH 7.5**

Tris	50 Mm
NaCl	150 mM
Distilled water	

- **TBST 0.1% (v/v)**

Tween 20	1 mL
TBS	1000 mL

- **TBST 0.05% (v/v)**

TBS	500 mL
TBST 0.1% (v/v)	500 mL

- **3 and 5% Blocking solution**

Dried fat-free milk powder	3 or 5 gm
Distilled water	100 ml

- **Chemiluminiscence solution**

Starting solution	1% (v/v)
Blotting substrate	

2.2.7.7. Stripping of the membranes

The stripping of the membranes was performed to remove primary and secondary antibodies from a western blot membrane and to allow the same membrane to be probed for different proteins with another antibody. In this regard, the membrane was soaked in methanol followed by washing with distilled water for 5 min. 0.2% NaOH solution was then added onto the membrane for 5 minutes over a shaker platform followed by washing the membrane with distilled water for 5 min. Then the membrane was blocked with a 5% blocking solution overnight at 4^{o}C. This was also followed by the addition of another primary antibody in a 3% blocking solution and the remaining protocol for the immunoblotting was similar to what was described previously.

3. Results

3.1. Expression of endothelins and their receptors in human lung fibroblasts

3.1.1. Expression of ET isoforms

To characterize the expression level of mRNA encoding for the different ET isoforms (ET-1, ET-2 and ET-3) in primary human lung fibroblasts (phLFb) and in the MRC-5 human lung fibroblast cell line, quantitative real time PCR was used. As shown in figure 3-1, under basal, un-stimulated conditions, phLFb, and MRC-5 fibroblasts, clearly express mRNA encoding ppET-1 and ppET-2, but phLFb expressed lower levels for ppET-1 and ppET-2 than MRC-5 cells. ppET-1 mRNA was found to be the major ET isoforms expressed, with mRNA levels about 100 times higher than those for ppET-2 in both cell types (Fig. 3-1). mRNA for ppET-3 was below detection limit in both, phLFb and MRC-5 cells.

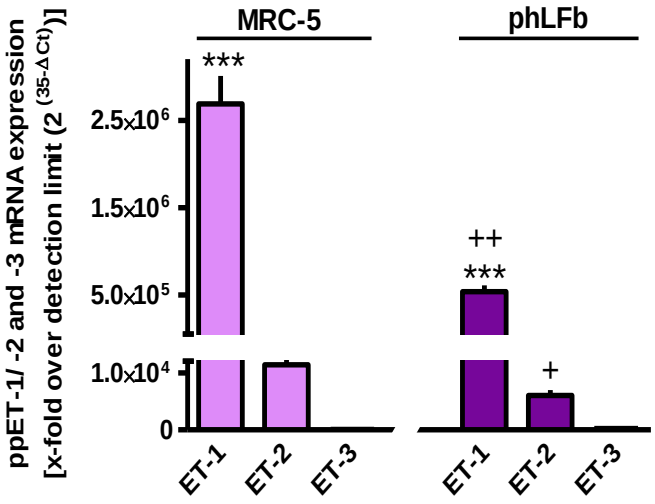


Figure 3-1: Expression of pp-ET-1, -2 and -3 in MRC-5 and primary (phLFb) human lung fibroblasts. Cells were grown in presence of 10% FCS in 35 mm culture dishes to confluency. Total RNA was isolated, treated with DNase and used for quantitative real time PCR with primers specific for the respective human sequences. *Height of columns:* mRNA expressed as n-fold over detection limit ($2^{(35-\Delta Ct)}$), given are means + S.E.M of n>6 independent experiments. Significance of differences: $^{***}P<0.001$ vs ET-2; $^{+}P<0.05$; $^{++}P<0.01$ vs respective value in MRC-5 cells.

3.1.2. Expression of ET receptor subtypes

ET-1 may act in an autocrine and paracrine fashion through binding to the GPCR (ET-A and ET-B) receptors. Thus, it was important to determine whether phLFb and MRC-5 fibroblasts express these receptors. Therefore, quantitative real time PCR was performed to determine the expression level of mRNA encoding for the two ET receptors subtypes (ET-A and ET-B) in phLFb and MRC-5 cells. As shown in figure 3-2, under basal, un-stimulated condition, mRNA encoding ET-A and ET-B receptors were clearly expressed in phLFb and in MRC-5 cells. In phLFb, the levels of mRNA for ET-B receptors were found to be significantly higher than those for ET-A receptors. However, there were no significant differences in the expression level of mRNA encoding ET-A and ET-B receptor in MRC-5 cells.

In addition, to provide evidence for the expression of ET-A and ET-B receptors at the protein levels, Western blot analysis was performed, using specific commercially available polyclonal antibodies for ET-A and ET-B receptors. As shown in figure 3-2B, the immunoblot result demonstrated one band for ET-B receptor of about 70 kDa, which is in the range of the expected molecular weight, and an additional protein band, with a lower molecular weight, was also detected. This additional protein band probably reflects a cellular degradation product which is recognized by the antibody. The immunoblot for ET-A receptor demonstrated also two bands, both with molecular weights lower than what was expected. Nonetheless, the larger band seems to be an ET-A receptor signal as it corresponds to a specific band of about 45 kDa seen in ET-A receptor transfected cells (antibody reference sheet of Santa Cruz).

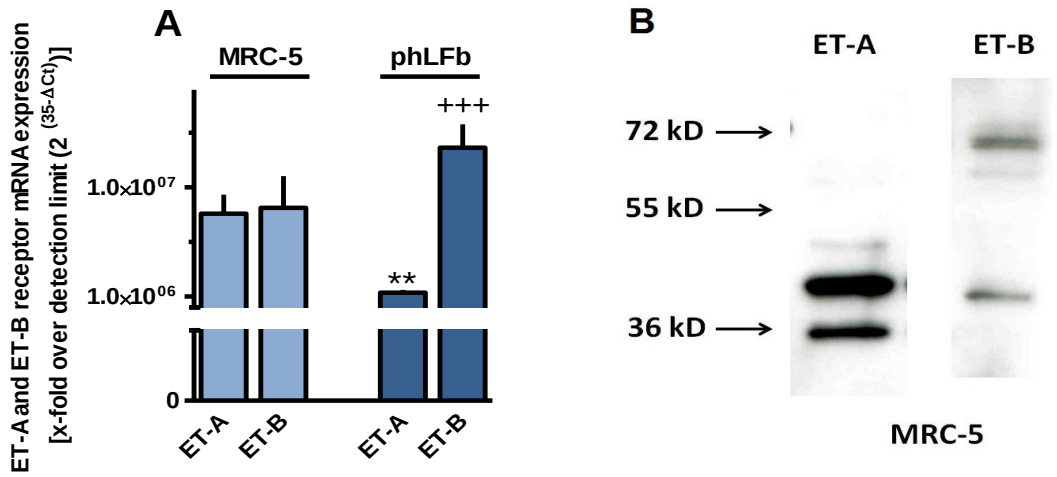


Figure 3-2: Expression of ET-A and ET-B receptors in MRC-5 and primary (phLFb) human lung fibroblasts. Cells were grown in presence of 10% FCS in 35 mm culture dishes to confluence. Either total RNA was isolated, treated with DNase and used for quantitative real time PCR with primers specific for the respective human sequences (A) or proteins were extracted and used for immunoblot analysis using commercially available antibodies against ET-A and ET-B receptor protein (B). A, *Height of columns:* mRNA expressed as n-fold over detection limit ($2^{(35-\Delta Ct)}$), given are means + S.E.M of n>6 independent experiments. Significance of differences: **P<0.01 vs respective value in MRC-5 cells; $^{+++}$P<0.001 vs ET-A (in phLFb). B: Representative samples of SDS PAGE of 4 similar experiments.

3.2. Regulation of ET expression and release in human lung fibroblasts

3.2.1. Effect of TGF-β on ET expression

In order to investigate whether the ET-1 gene expression in phLFb and MRC-5 cells is regulated by TGF-β, the effect of TGF-β on the expression of ppET-1 transcription was studied. As depicted in figure 3-3A, TGF-β induced in concentration-dependent manner a strong, sustained and selective increase in ppET-1 mRNA expression in both phLFb and MRC-5 cells. Furthermore, 5 ng/ml TGF-β did not exhibit any stronger effect than that obtained with 1 ng/ml TGF-β, as maximum effective concentration on the expression of ppET-1 in human lung fibroblasts. A marked stimulatory effect of TGF-β on ppET-1 transcription was already observed within one hour (Fig. 3-5A and 3-5B) and remained unchanged until one week of exposure (Fig. 3-3A). On the other hand, after 24 hours treatment of phLFb and MRC-5 cells with 1 ng/ml TGF-β, ppET-2 mRNA expression was decreased (Fig. 3-3B) and ppET-3 mRNA expression remained below detection limit (n=9, data not shown).

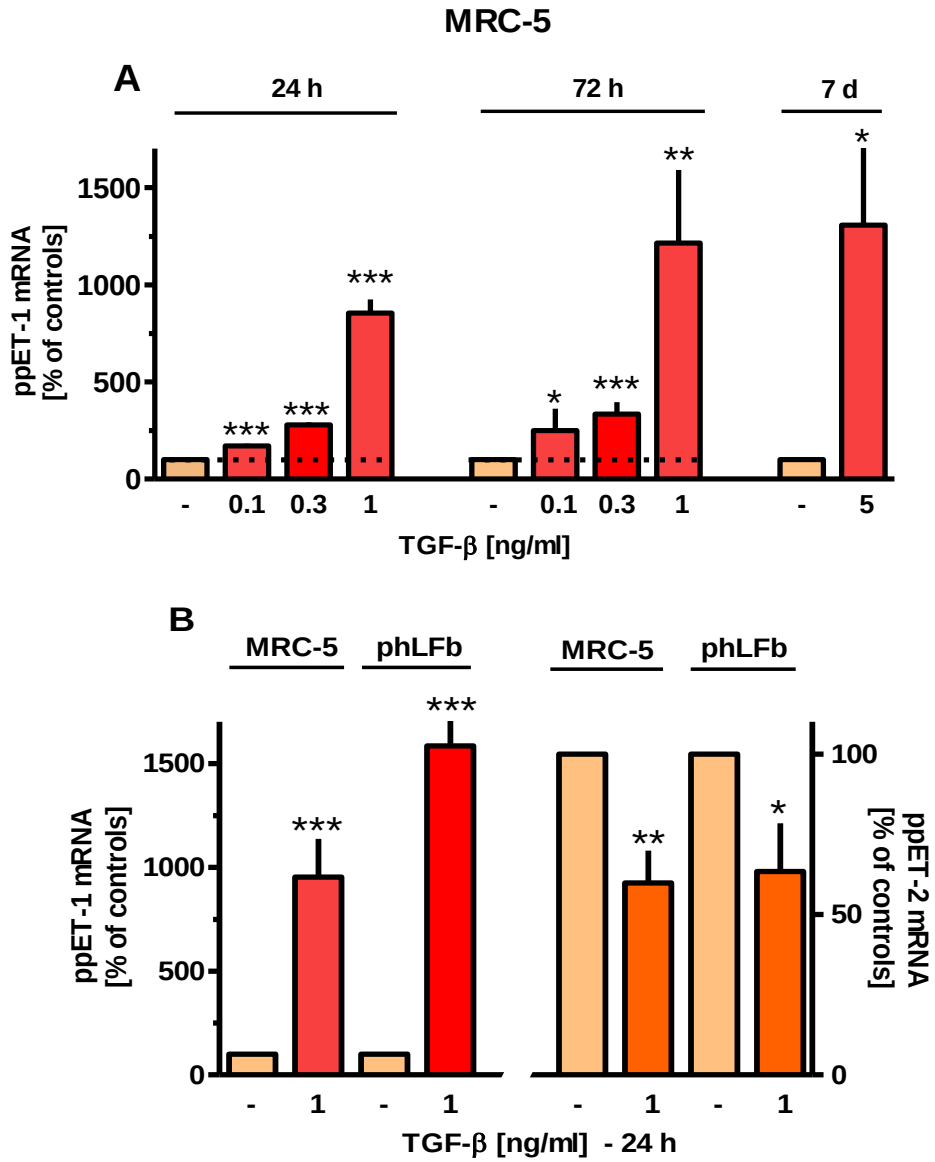


Figure 3-3: Effects of TGF-β on expression of ppET-1 and ppET-2 in MRC-5 and primary (phLFb) human lung fibroblasts. After dissemination, cells were cultured for 24 h in presence of 10% FCS followed by 24 or 72 h or 7 d (A, as indicated) or 24 h (B) in presence of 1% FCS and absence or presence of TGF-β (0.1 - 5 ng/ml). Thereafter, total RNA was isolated, treated with DNase and used for quantitative real time PCR. *Height of columns:* ppET-1 or -2 mRNA (ΔCt over GAPDH) expressed as % of the control of the individual cell preparation, given are means + S.E.M of n≥6 independent experiments. Significance of differences: *P<0.05; **P<0.01; ***P<0.001 vs respective value in absence of TGF-β.

3.2.2. Effect of TGF-β on ppET-1 mRNA stability and/or transcription

In order to examine whether TGF-β has an effect on ppET-1 mRNA stability and/or transcription, transcription inhibitor actinomycin D (Act, 30 µM) was used to block the synthesis of new transcript, thus allowing quantification of the rate of decay

of ppET-1 mRNA. MRC-5 fibroblasts were incubated for 0 to 120 min with 30 µM of Act alone, or Act and TGF-β (1 ng/ml) in combination. As shown in figure 3-4A, after inhibition of *de-novo* RNA synthesis by 30 µM actinomycin D, ppET-1 mRNA showed a rapid decline, with a half life of about 30 min. Furthermore, the stimulatory effect induced by a 60 minute exposure to 1 ng/ml TGF-β on ppET-1 mRNA was prevented by pre-treatment with 30 µM Act (Fig. 3-4B). This suggests that TGF-β had no effect on the rate of decay of ppET-1 transcripts.

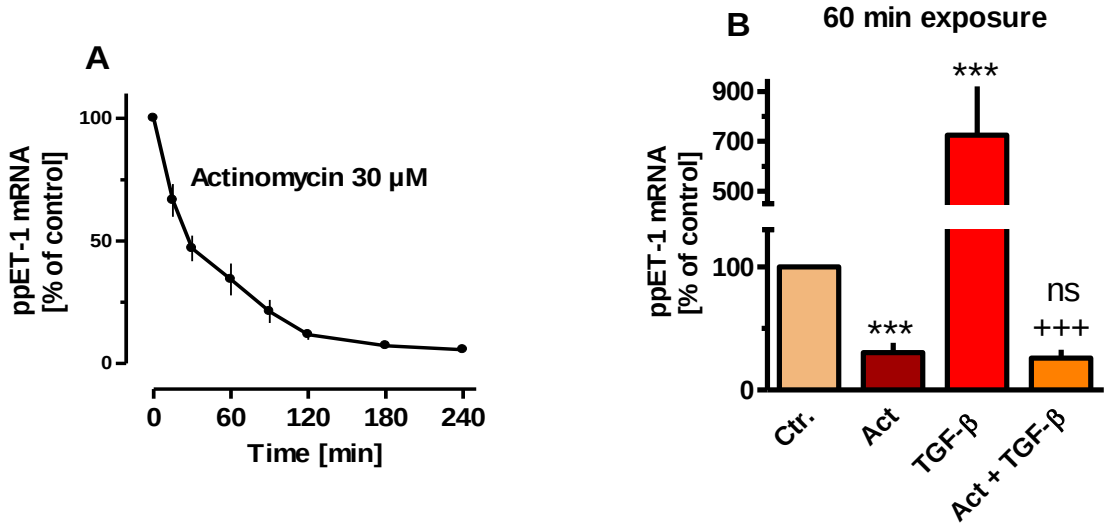


Figure 3-4: Time-dependent effects of actinomycin D (30 µM, A) and effect TGF-β (1 ng/ml, 1 h) in absence and presence of actinomycin D (Act, present 15 min before TGF-β, B) on expression of ppET-1 in MRC-5 human lung fibroblasts. After dissemination, cells were cultured for 24 h in presence of 10% FCS followed by up to 4 h in presence of 1% FCS and absence or presence test drugs. Thereafter, total RNA was isolated, treated with DNase and used for quantitative real time PCR. *Ordinate (A) and height of columns (B):* ppET-1 mRNA (ΔCt over GAPDH) expressed as % of the Ctr. of the individual cell preparation, given are means + S.E.M of n≥4 independent experiments. Significance of differences: ***P<0.001 vs Ctr.; $^{+++}$P<0.001 vs value in presence of TGF-β alone; ns, not significant vs Act alone.

In a further series of experiments, cycloheximide, a protein synthesis inhibitor, was used to assess whether ppET-1 mRNA stimulation by TGF-β is induced through de-novo cellular protein synthesis. As shown in figure 3-5A, cycloheximide induced a rapid and strong up-regulation of ppET-1 mRNA expression of about tenfold within one and a half hours and more than fourteen-fold after four and a half hours. Strikingly, the stimulatory effect of TGF-β was highly augmented in the presence of 30 µM cycloheximide, resulting in a more than thirty five-fold increase in ppET-1 mRNA after one and four hours exposure to TGF-β in presence of cycloheximide (Fig. 3-5B).

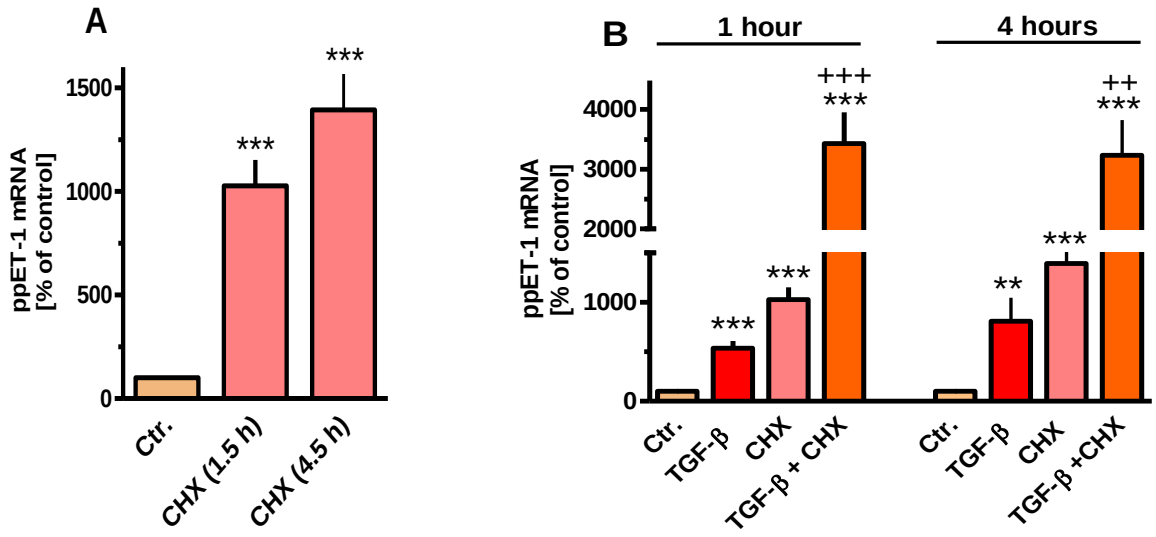
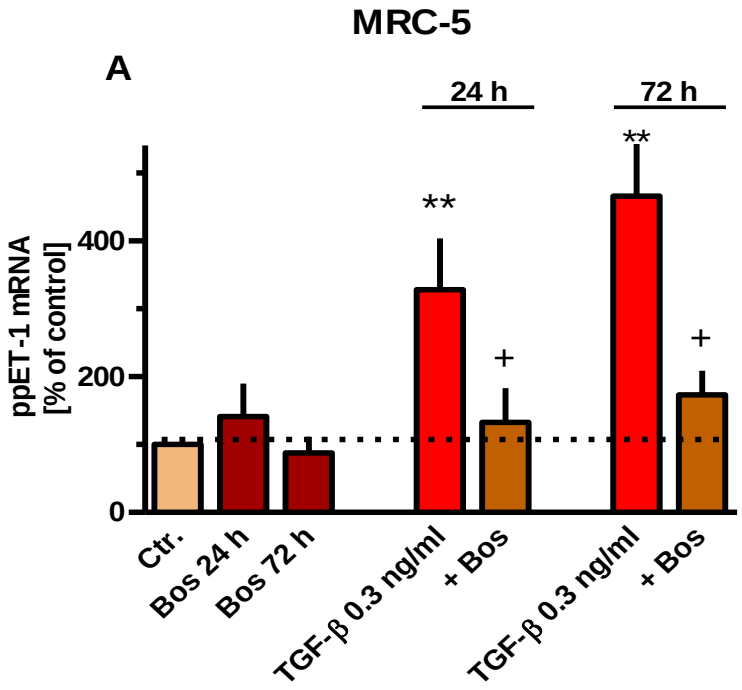


Figure 3-5: Effects of cycloheximide (CHX, A) or TGF-β in absence or presence of CHX (B) on expression of ppET-1 in MRC-5 human lung fibroblasts. After dissemination, cells were cultured for 24 hours in presence of 10% FCS followed by 1.5 or 4.5 hours in presence of 1% FCS and absence or presence of CHX (30 µM) or 1 hour and 4 hours TGF-β (1 ng/ml) and/or CHX (30 µM, 1.5 h and 4.5 h, i.e. present 30 minute before TGF-β). Thereafter, total RNA was isolated, treated with DNase and used for quantitative real time PCR. *Height of columns:* ppET-1 mRNA (ΔCt over GAPDH) expressed as % of the Ctr. of the individual cell preparation, given are means + S.E.M of n≥6 independent experiments. Significance of differences: **P<0.01 ***P<0.001 vs respective Ctr.; $^{++}$P<0.01, $^{+++}$P<0.001 vs respective value in presence of TGF-β or CHX alone.

3.2.3. Role of ET receptors in TGF-β-induced ppET-1 mRNA expression

In order to determine the role of ET receptors in TGF-β mediated up-regulation of ppET-1 mRNA expression in MRC-5 cells, interaction experiments with two selective ET-receptor antagonists BQ-123 (ET-A) (Ihara *et al.,* 1992) and BQ-788 (ET-B) (Ishikawa *et al.,* 1994), and a non-selective ET receptor antagonist (bosentan) (Clozel *et al.,* 1994) were performed. The effects of ET receptor antagonists on TGF-β induced up-regulation of ppET-1 mRNA expression were studied at two time points (24 hours and 72 hours). As shown in figures 3-6A, B and C, none of the ET-1 antagonists had any effect on the basal levels of ppET-1 mRNA expression in MRC-5 cells, neither after 24 hours nor 72 hours of exposure. However, the stimulatory effect caused by 24 hours exposure to a sub-maximally effective concentration of TGF-β (0.3 ng/ml) was inhibited by pre-treatment with 10 µM bosentan (Fig. 3-6A)) as well as with 1 µM BQ-123, the selective ET-A receptor

antagonist (120 % of controls, n=6, Fig. 3-6B), whereas it was not affected by the selective ET-B antagonist (BQ-788, 100nM, Fig. 3-6C). The increase in pp-ET-1 mRNA expression caused by 1 ng/ml TGF-β, a maximally effective concentration, was neither significantly affected by the selective ET-A antagonist BQ-123 (1 µM, by about 800%, n=11), nor by the selective ET-B antagonist (BQ-788, 100 nM, to about 850%, n=6 (Fig. 3-6B and C)). Furthermore, the up-regulation of ppET-1 transcription induced by 0.3 ng/ml TGF-β at 72 hours exposure of MRC-5 cells was strongly inhibited by 10 µM bosentan and BQ-123 (1 µM) but was not affected by BQ-788 (100 nM). The increase in ppET-1 mRNA expression caused by 1 ng/ml TGF-β at 72 hours exposure was neither affected by BQ-123 (1 µM, by about 900%) nor BQ-788 (100 nM, by about 800%).



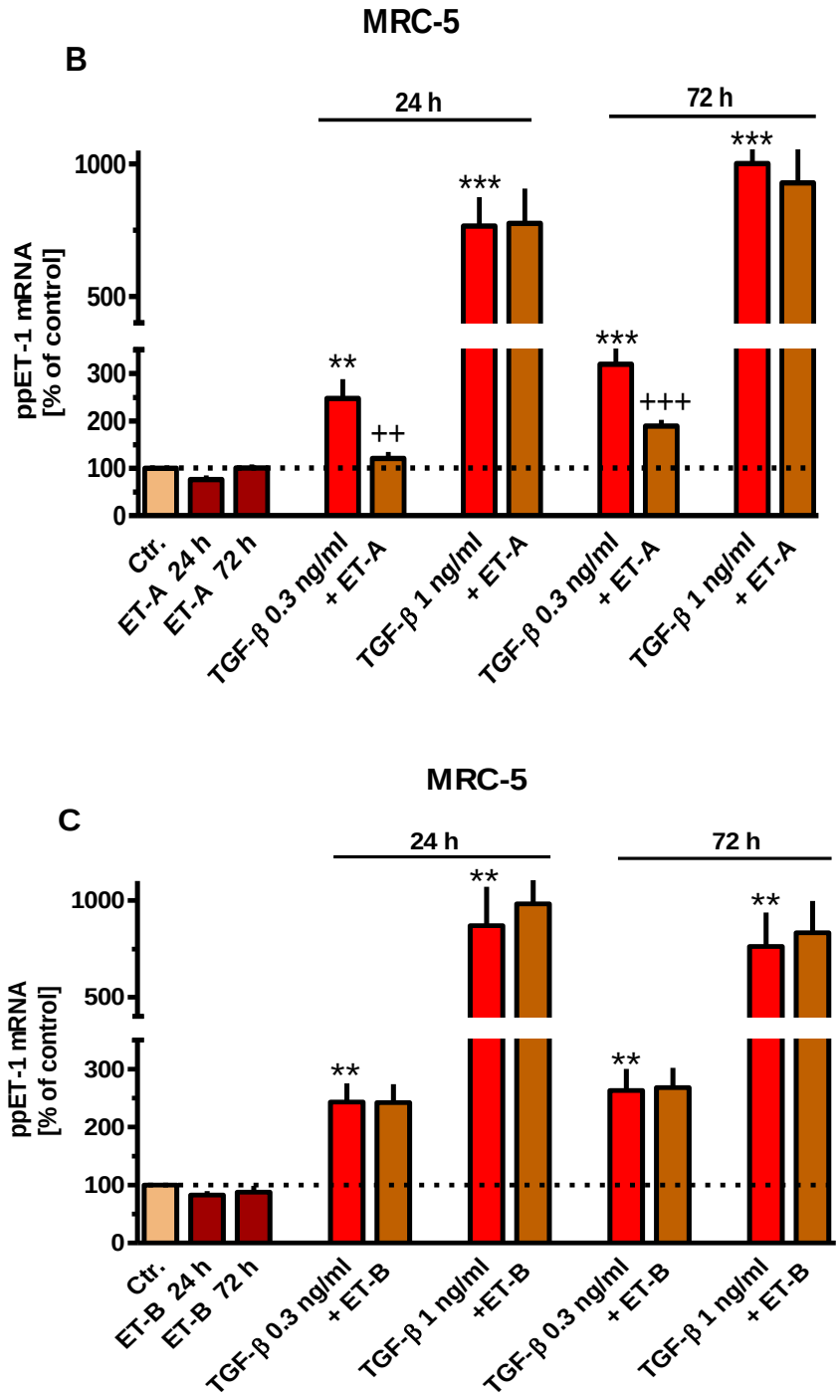
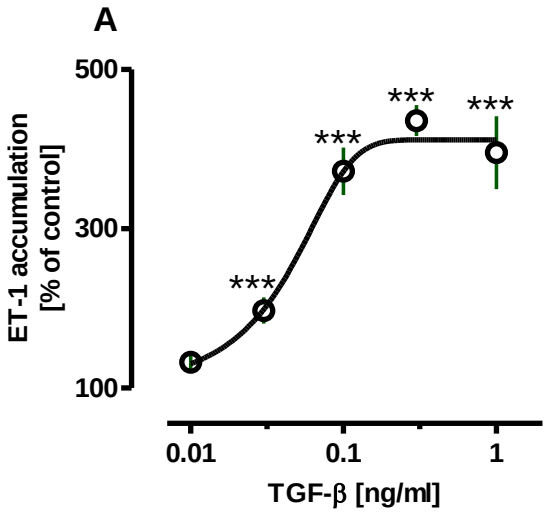


Figure 3-6: Effects of TGF-β in absence and presence of bosentan (Bos), the ET-A (BQ123) or ET-B (BQ-788) receptor selective antagonist on expression of ppET-1 mRNA in MRC-5 human lung fibroblasts. After dissemination, cells were cultured for 24 h in presence of 10% FCS followed by 24 or 72 h in presence of 1% FCS and absence or presence of 0.3 or 1 ng/ml TGF-β and/or 10 µM Bos, 1 µM BQ-123 or 100 nM BQ-788. Thereafter, total RNA was isolated, treated with DNase and used for quantitative real time PCR. *Height of columns:* ppET-1 (ΔCt over GAPDH) expressed as % of the Ctr. of the individual cell preparation, given are means + S.E.M of n≥6. Significance of differences: **P<0.01, ***P<0.001 vs Ctr.; $^{+}$P<0.05, $^{++}$P<0.01, $^{+++}$P<0.001 vs respective value in presence of antagonist alone.

3.2.4. Effects of TGF-β and ET receptor antagonist on ET-1 release

In order to confirm that the induction of ET-1 mRNA by TGF-β results in increased synthesis and release of ET-1, the levels of ET-1 in the culture medium of MRC-5 cells was measured by a specific ELISA. Under controlled conditions, ET-1 accumulation in the culture medium of MRC-5 cells amounted to 6.8 ± 0.3 pg/ml (≈ 3 nM) at the end of a 6 hours incubation period, corresponding to 44.8±2.6 pg/mg protein (n=74). Addition of TGF-β evoked in a concentration-dependent manner, a strong increase in ET-1 accumulation (Fig. 3-7A), resulting in a maximal accumulation of 34 ± 7 pg/ml ET-1 (≈ 15 nM) in presence of 0.3 ng/ml TGF-β (n=17). The maximum effect on ET-1 accumulation caused by 0.1 - 0.3 ng/ml TGF-β was a three- to fourfold increase. Thus, the TGF-β-induced increase in expression of ET-1 mRNA was accompanied by an increase in ET-1 synthesis and release. Interestingly, the concentrations of TGF-β (0.1-0.3 ng/ml), that caused maximal increase in ET-1 accumulation were lower than those required for maximal effect on ppET-1 mRNA expression (1 ng/ml, Fig. 3-3). In other words, the further substantial increase in mRNA seen at 1 ng/mg TGF-β did not result in an equivalent further increase in ET-1 accumulation. Nonetheless, in line with observations on mRNA levels, the up-regulation of ET-1 accumulation by a sub-maximally effective concentration of TGF-β was also significantly attenuated by bosentan (Fig.3-7B).



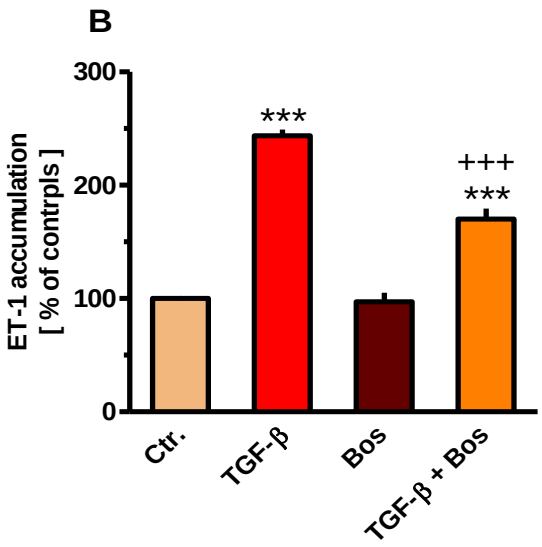


Figure 3-7: Concentration-dependent effects of TGF-β (A) and effect of 0.03 ng/ml TGF-β in absence and presence of bosentan (Bos, 10 µM) (B) on release of ET-1 by MRC-5 human lung fibroblasts. After dissemination, cells were cultured for 24 hours in presence of 10% FCS followed by 48 hours in presence of 1% FCS and absence or presence of test substances as indicated. The last medium change was performed 6 hours before the supernatant were collected for ET-1 determination by ELISA. ET-1 accumulation expressed as % of the Ctr. of the individual cell preparation, given are means + S.E.M of n≥12 independent experiments. Significance of differences: ***P<0.001 vs respective Ctr.; $^{+++}$P<0.001 vs respective value in presence of TGF-β or Bos alone.

In order to test whether ET converting enzyme activity could have been a limiting factor for the accumulation of ET-1 under the present culture conditions, the conversion of big-ET-1 into ET-1 was studied. Addition of big-ET-1 to confluent cell cultures resulted in concentration- and time-dependent accumulation of ET-1 in the supernatant. Maximal rate of conversion appears to occur at a concentration of 100 nM big-ET-1, and steady-state conditions (where formation and degradation might be balanced) appear to be reached in 6 hours (Fig. 3-8). Notably, the ET-1 concentrations during 6 hours incubation with 100 nM big-ET-1 were about 40 times higher than those observed during a 6 hour collection period after exposure to maximally stimulating concentrations of TGF-β. Pre-treatment of the cells with TGF-β (0.3 ng/ml for 48 hours) did not affect the accumulation of ET-1 during 3 or 6 hours of incubation with 1, 10 or 100 nM big-ET-1 (data not shown, each point n≥6).

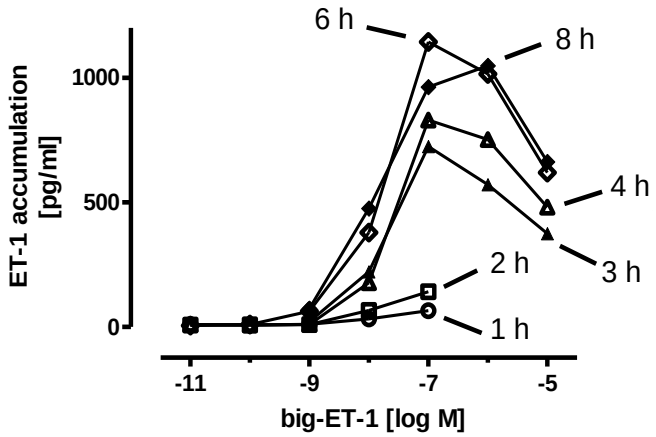


Figure 3-8: Time- and concentration-dependent conversion of big-ET-1 into ET-1 by MRC-5 human lung fibroblasts in culture. Big-ET-1 at the concentrations given was added to nearly confluent cultures in absence of FCS for 1 to 8 hours, as indicated, followed by determination of ET-1 accumulation in the supernatant. Given are means of n≥6 independent experiments, for clarity of the figure without S.E.M, which were between 5 and 15% of the respective mean value.

3.2.5. Role of canonical (Smad) and non-canonical (MAPK, Rho G-protein) signaling in TGF-β-induced ppET-1 mRNA expression

3.2.5.1. Role of Smad3 in TGF-β-mediated ppET-1 mRNA expression

To investigate the role of Smad3 pathway in TGF-β-mediated increases in ppET-1 mRNA expression in MRC-5 fibroblasts, interaction experiments with a specific inhibitor of the canonical TGF-β effector Smad3, Smad3 inhibitor (SIS3) were performed. MRC-5 fibroblasts were cultured for 1 and 4 hours in presence of 1% FCS and in absence or presence of 1 ng/ml TGF-β and/or 10 µM SIS3. Real time PCR analysis revealed that TGF-β (1 ng/ml, a maximally effective concentration) induced a rapid and strong increase in ppET-1 mRNA expression within one and four hours by about 570% and 470%, respectively (Fig. 3-9A and B). Whereas the Smad 3 inhibitor (SIS3) provoked a strong reduction of the basal level of ppET-1 mRNA expression by about 65% and 75% after 1 and 4 hour, respectively (Fig. 3-9A and 5B). Moreover, the increase in ppET-1 mRNA expression induced by 1 and 4 hours exposure to 1 ng/ml TGF-β was largely attenuated by SIS3 (Fig. 3-9A and B).

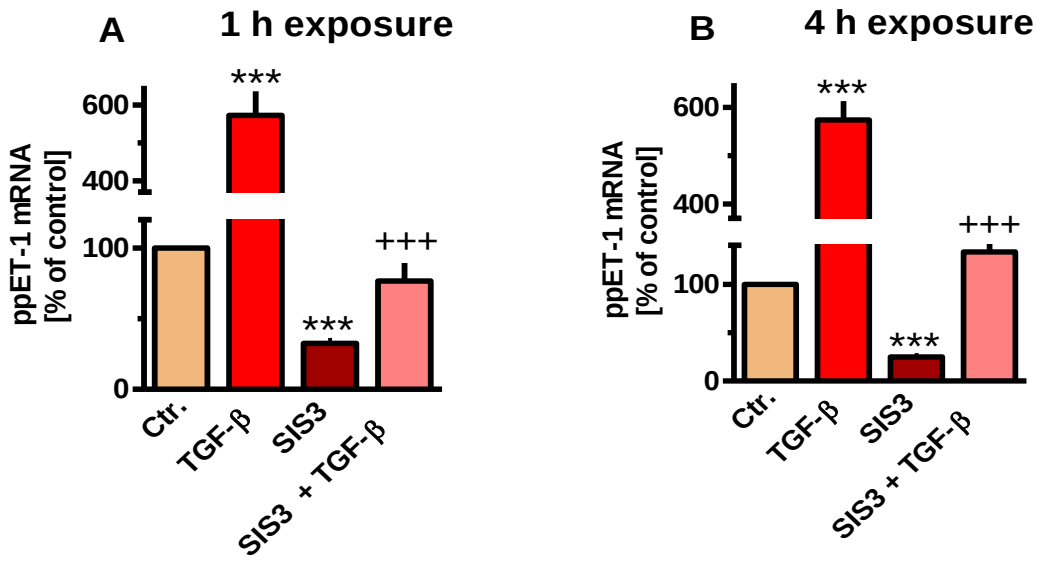


Figure 3-9: Effects of TGF-β in absence or presence of the smad3 inhibitor (SIS3) on expression of ppET-1 in MRC-5 human lung fibroblasts. After dissemination, cells were cultured for 24 h in presence of 10% FCS followed by 1 h or 4 h in presence of 1% FCS and absence or presence of 1 ng/ml TGF-β and/or 10 µM SIS3 (present 30 min before TGF-β). Thereafter, total RNA was isolated, treated with DNase and used for quantitative real time PCR. *Height of columns*: ppET-1 (ΔCt over GAPDH) expressed as % of the Ctr. of the individual cell preparation, given are means + S.E.M of n≥5. Significance of differences: ***P<0.001 vs Ctr.; $^{+++}$P<0.001 vs value in presence of SIS3 or TGF-β alone.

3.2.5.2. Role of the ERK MAPK pathway in TGF-β-induced ppET-1 mRNA expression

In order to investigate the role of ERK MAPK pathway in TGF-β-mediated increases in ppET-1 mRNA expression in MRC-5 fibroblasts, interaction experiments with PD 098059, a specific inhibitor of MAPK-activating enzyme (MEK) (Alessi *et al.,* 1995; Dudley *et al.,* 1995), were performed. Exposure of the cells for four hours and eight hours to 10 and 30 µM PD 098059 had no effect on the ppET-1 mRNA expression level (data not shown). However, in 24 hours exposure to PD 098509 (10 and 30 µM) induced in a concentration dependent manner clear increase in the ppET-1 mRNA expression (2.5-fold and 3.7-fold, respectively) (Fig. 3-10). The stimulatory effect of 0.3 ng/ml TGF-β was highly augmented in presence of PD 098509 (10 and 30 µM), resulting in a more than five-fold and thirteen-fold increase in ppET-1 mRNA, respectively, after 24 hours (Fig. 3-10).

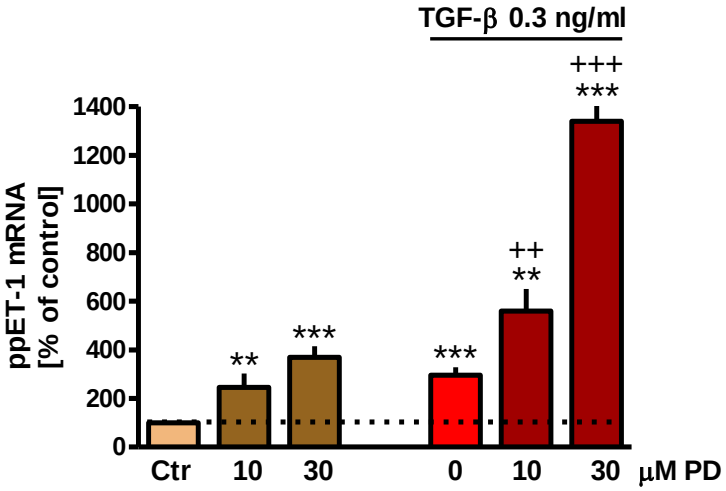


Figure 3-10: Effects TGF-β in absence or presence of PD 098059 (PD) on expression of ppET-1 in MRC-5 fibroblasts. After dissemination, cells were cultured for 24 hours in presence of 10% FCS followed by 24 hours in presence of 1% FCS and absence or presence of TGF-β (0.3 ng/ml) and/or PD (10 or 30 µM). Thereafter, total RNA was isolated, treated with DNase and used for quantitative real time PCR. *Height of columns:* ppET-1 mRNA (ΔCt over GAPDH) expressed as % of the Ctr. of the individual cell preparation, given are means + S.E.M of n≥7 independent experiments. Significance of differences: **P<0.01, ***P<0.001 vs respective Ctr.; $^{++}$P<0.001, $^{+++}$P<0.001 vs respective value in presence of TGF-β or PD alone.

3.2.5.3. Role of the Rho-kinase on TGF-β-induced ppET-1 mRNA expression

In order to test whether Rho kinase might be involved in the regulation of ppET-1 mRNA by TGF-β in MRC-5 human lung fibroblast, interaction experiments with a specific inhibitor of Rho-kinase, Y-27632 (Ishizaki *et al.,* 2000; Shimokawa *et al.,* 2007) were performed. As shown in figure 3-11, TGF-β, in a sub-maximally effective concentration of 0.3 ng/ml exerted again a clear stimulatory effect on the ppET-1 mRNA expression by about 390% was observed, whereas the Rho-kinase inhibitor (Y-27632) caused a significant reduction of the ppET-1 mRNA levels by about 30% and 50% at 3 µM and 10 µM, respectively. However, in presence of Y-27632, the stimulatory effect of 0.3 ng/ml TGF-β on ppET-1 mRNA expression was unaffected. When expressed in relation to the respective value of 3 and 10 µM Y-27632, the increase caused by TGF-β was by about 465% and 490%, respectively.

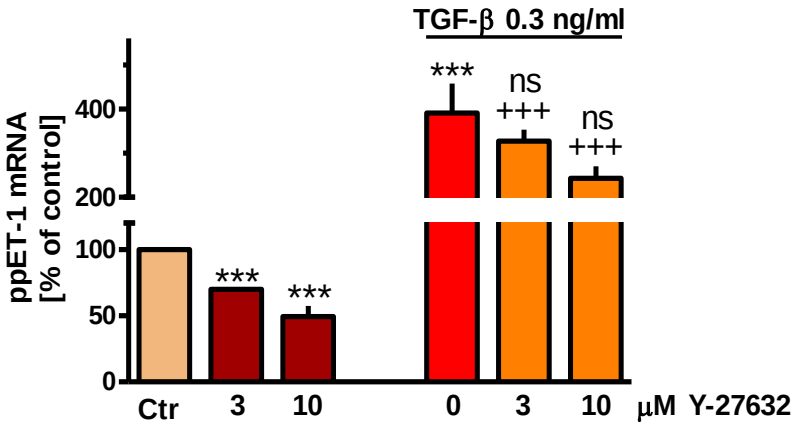


Figure 3-11: Effects of TGF-β in absence or presence of the Rho-kinase inhibitor Y27632 on expression of ppET-1 in MRC-5 human lung fibroblasts. After dissemination, cells were cultured for 24 hours in presence of 10% FCS followed by 24 hours in presence of 1% FCS and absence or presence of 0.3 ng/ml TGF-β and/or (3 or 10 μM) Y-27632 (present 30 minute prior TGF- β). Thereafter, total RNA was isolated, treated with DNase and used for quantitative real time PCR. *Height of columns:* ppET-1 (ΔCt over GAPDH) expressed as % of the Ctr. of the individual cell preparation, given are means + S.E.M of n=5 independent experiments. Significance of differences: ***P<0.001 vs Ctr.; $^{+++}$P<0.001 vs value in presence of Y-27632 alone; ns, not significant vs TGF-β alone.

3.2.6. Role of β_2-adrenoceptors and muscarinic receptors in the ET-1 expression

3.2.6.1. Effect of β_2-adrenoceptor agonist on the ET-1 expression

β_2-Adrenoceptors have an important role in regulating various cellular functions in lung fibroblasts, including proliferation and collagen synthesis. To investigate whether the expression of ppET-1 mRNA in human lung fibroblasts is regulated by β-adrenergic mechanism, a highly selective, long-acting β_2-adrenoceptor agonist, olodaterol was used. As shown in figure 3-12, exposure of the cells to olodaterol for 24 hours caused a clear reduction in the expression of ppET-1 mRNA. An inhibition by 45% was observed at 10 nM and 100 nM, whereas 1 nM olodaterol had no effect on the ppET-1 mRNA expression. As 10 nM olodaterol appeared to be the maximum effective concentration, a concentration of 10 nM was used in all subsequent experiments.

Furthermore, to determine the selectivity of olodaterol, interaction experiments with highly selectivity antagonists were performed using the β_2-adrenoceptor selective antagonist ICI 118,551 (1 µM) and the β_1-adrenoceptor selective antagonist CGP 20712 (3 µM) (Baker, 2005). As depicted in figure 3-12, ICI 118,551 (1 µM) and CGP 20712 (3 µM) did not affect the basal levels of ppET-1 mRNA expression in MRC-5 fibroblasts. However, the inhibitory effect of 10 µM olodaterol was inhibited by the β_2-adrenoceptor selective antagonist ICI 118,551, but not affected by the β_1-selective antagonist CGP 20712 (Fig. 3-12).

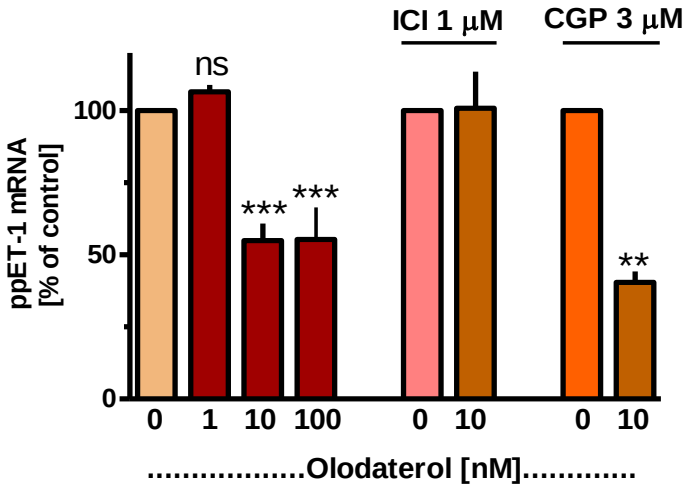


Figure 3-12: Effects of olodaterol in absence or presence of the β_2-adrenoceptor selective antagonist ICI 118,551 (ICI, 1 µM) or the β_1-adrenoceptor selective antagonist CGP 20712 (CGP, 3 µM) on expression of ppET-1 mRNA in MRC-5 human lung fibroblasts. After dissemination, cells were cultured for 24 h in presence of 10% FCS followed by 24 hours in presence of 1% FCS and in absence or presence of test drugs, the antagonists being present 30 minute prior to olodaterol. Thereafter, total RNA was isolated, treated with DNase and used for quantitative real time PCR. *Height of columns:* ppET-1 (ΔCt over GAPDH) expressed as % of the respective control (in absence or presence of antagonists) of the individual cell preparation, given are means + S.E.M of n=5 independent experiments. Significance of differences: **P<0.01, ***P<0.001 vs respective control; ns, not significant vs respective Ctr..

3.2.6.2. Effect of β_2-adrenoceptor agonist on TGF-β-mediated ET-1 expression

As TGF-β was shown to up-regulate and β_2-adrenoceptor to reduce ET-1 gene expression, possible interactions between TGF-β and β_2-adrenoceptor agonist were studied. As shown in figure 3-13A, the increase in ppET-1 mRNA expression caused by 24 hours exposure to 0.3 ng/ml TGF-β, a sub-maximally effective

concentration, was effectively opposed by 10 and 100 nM olodaterol, but not significantly affected by 1 nM olodaterol. This inhibitory effect of 10 and 100 nM olodaterol in presence of 0.3 ng/ml TGF-β (reduction by about 46% and 38% respectively) was comparable to that observed in absence of TGF-β (compare Figs. 3-12 and 3-13A). However, the increase in ppET-1 mRNA expression caused by 24 hours exposure to TGF-β, a maximally effective concentration of 1 ng/ml was not significantly affected by 10 or 100 nM olodaterol. i.e., in presence of 1 ng/ml TGF-β, the inhibitory effect of the β_2-agonist was abolished (Fig. 3-13B).

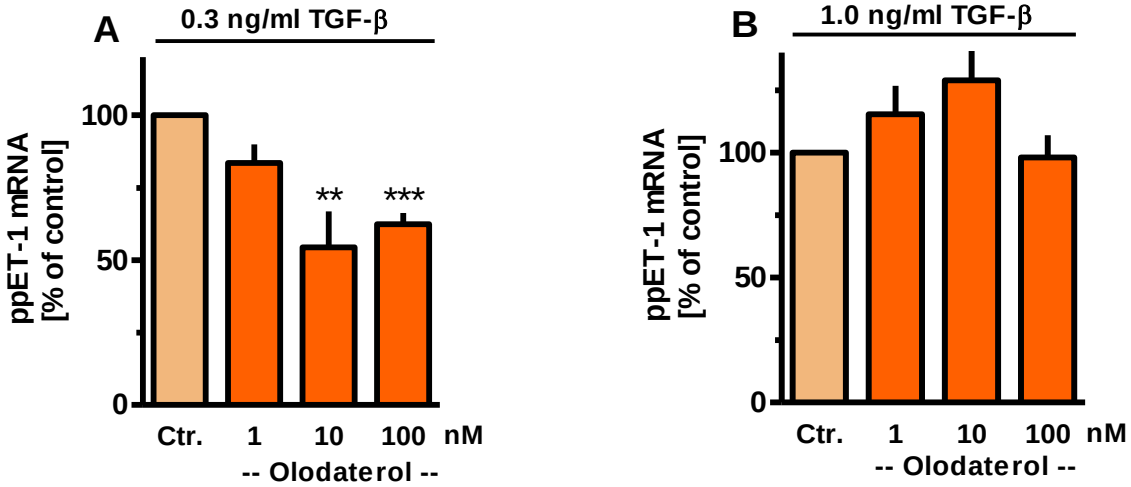


Figure 3-13: Effects of olodaterol in absence or presence of 0.3 (A) or 1 ng/ml TGF-β (B) on expression of ppET-1 in MRC-5 human lung fibroblasts. After dissemination, cells were cultured for 24 hours in presence of 10% FCS followed by 24 hours in presence of 1% FCS and absence or presence of test substances at the concentrations given, olodaterol being present 30 min prior to TGF-β. Thereafter, total RNA was isolated, treated with DNase and used for quantitative real time PCR. *Height of columns:* ppET-1 (ΔCt over GAPDH) expressed as % of the Ctr. (presence of TGF-β alone) of the individual cell preparation, given are means + S.E.M. of n=5 independent experiments. Significance of differences: **P<0.01; ***P<0.001 vs Ctr..

3.2.6.3. Role of cyclic AMP effectors (PKA and Epac) in ET-1 expression

Intracellular cAMP regulates different cellular functions, mainly through two downstream targets, PKA (protein kinase A) and Epac (Exchange protein directly activated by cAMP). To test whether the expression of ppET-1 mRNA in MRC-5 fibroblasts is regulated by these intracellular targets (PKA and Epac) of the second messenger cAMP, the cAMP analogs 6-Bnz-cAMP and 8-CPT-2'-O-Me-cAMP, known to preferentially activate PKA and Epac, respectively (Holz *et al.,* 2008;

Poppe *et al.,* 2008) were used. As illustrated in figure 3-14A, the selective protein kinase A agonist (6-Bnz-cAMP, 500 µM) caused a substantial reduction in ppET-1 mRNA expression by 63%, whereas the selective Epac agonist (8-CPT-2′-O-Me-cAMP, 100 µM) caused only a marginal inhibition by 22%. The inhibitory effect of the selective β_2-adrenoceptor agonist oldaterol was fully mimicked by the effect of the selective protein kinase A (PKA) agonist (Fig. 3-14).

Furthermore, the impact of the cyclic AMP (cAMP) effectors PKA and Epac in TGF-β-mediated increase in expression of ppET-1 mRNA in MRC-5 fibroblasts was also studied. As shown in figure 3-14B, in presence of 0.3 ng/ml TGF-β activation of either PKA or Epac with selective PKA-agonist 6-Bnz-cAMP (500 µM) and selective Epac-agonist 8-CPT-2'-O-Me-cAMP (100 µM) respectively, caused a decrease in ppET-1 mRNA expression comparable to that in absence of 0.3 ng/ml TGF-β. However, in presence of the maximum effective concentration of TGF-β, neither the PKA-agonist nor the Epac-agonist affected ppET-1 mRNA expression (Fig. 3-14C).

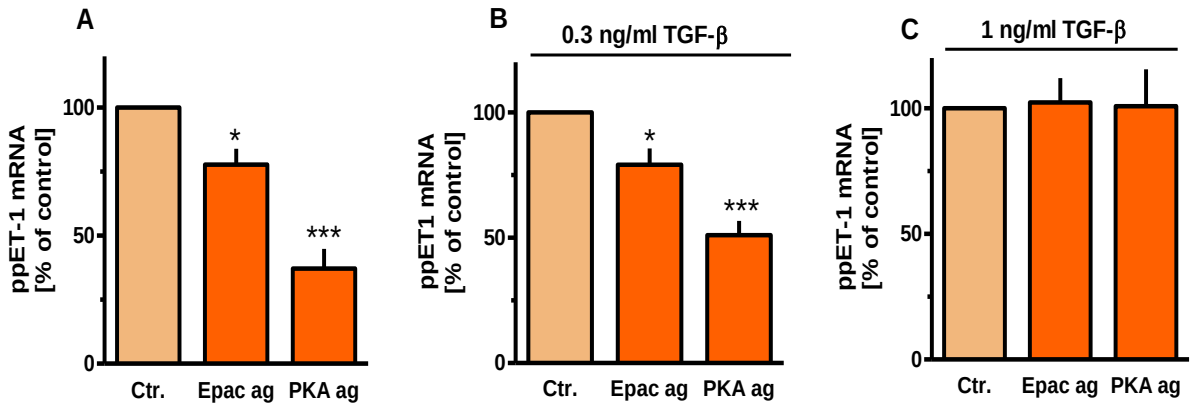


Figure 3-14: Effects of the selective PKA (6-Bnz-cAMP, 500 µM) or Epac (8-CPT-2'-O-Me-cAMP, 100 µM) agonist in absence or presence of 0.3 or 1 ng/ml TGF-β on expression of ppET-1 in MRC-5 human lung fibroblasts. After dissemination, cells were cultured for 24 h in presence of 10% FCS followed by 24 hours in presence of 1% FCS and absence or presence of test substances at the concentrations given, the selective PKA and Epac agonists being present 30 min prior to TGF-β. Thereafter, total RNA was isolated, treated with DNase and used for quantitative real time PCR. *Height of columns:* ppET-1 (ΔCt over GAPDH) expressed as % of the respective Ctr. or presence of TGF-β alone of the individual cell preparation, given are means + S.E.M. of n=5 independent experiments. Significance of differences: *P<0.05; ***P<0.001 vs respective Ctr..

3.2.6.4. Effect of muscarinic cholinergic receptor agonist on the ET-1 expression

In order to investigate, whether the muscarinic cholinergic receptor is involved in the regulation of ppET-1 mRNA in MRC-5 fibroblasts, effects of the muscarinic agonist oxotremorine (Ringdahl *et al.,* 1983) and the muscarinic antagonist tiotropium were studied. As shown in figure 3-15, exposure of the cells for 24 hours to oxotremorine (10 µM, previously shown to be a maximally effective concentration in eliciting muscarinic effects in MRC-5 cells, Matthiesen *et al*., 2006; Haag *et al.*, 2008a), caused about three-fold increase in the expression of ppET-1 mRNA. This stimulatory effect of oxotremorine was prevented by the muscarinic antagonist tiotropium (10 nM), which alone had no effect on ppET-1 transcription.

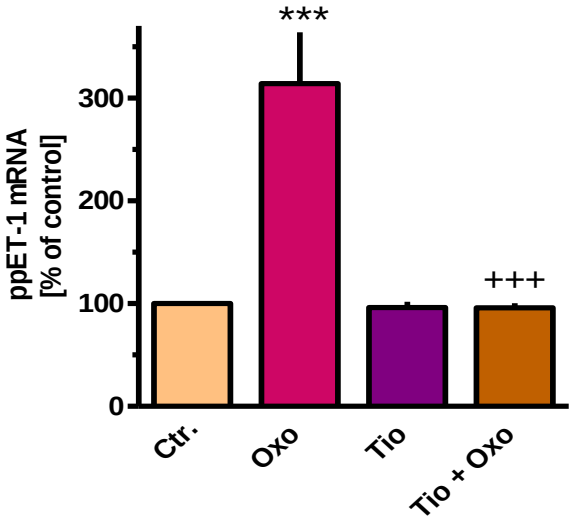


Figure 3-15: Effects of oxotremorine (Oxo, 10 µM) and/or tiotropium (Tio, 10 nM) on expression of ppET-1 in MRC-5 human lung fibroblasts. After dissemination, cells were cultured for 24 hours in presence of 10% FCS followed by 24 h in presence of 1% FCS and in absence of presence test drugs, Tio being present 30 min prior to Oxo. Thereafter, total RNA was isolated, treated with DNase and used for quantitative real time PCR. *Height of columns:* ppET-1 (ΔCt over GAPDH) expressed as % of the Ctr. of the individual cell preparation, given are means + S.E.M. of n≥4 independent experiments. Significance of differences: ***P<0.001 vs Ctr.; $^{+++}$P<0.001 vs Oxo alone.

Since β_2-adrenoceptors and muscarinic receptors appear to mediate opposing effect on the expression of ET-1 gene (see figures 3-12 and 3-15), possible interaction between olodaterol and oxotremorine on ET-1 gene expression were examined. The results shown in figure 3-16 indicate that the stimulatory effect of oxotremorine (10 µM) on ppET-1 mRNA expression was strongly inhibited by

olodaterol (10 nM). In presence of both drugs, no significant increase by 14 ± 7% in ppET-1 mRNA level in comparison to the control was observed. However, in presence of both drugs, ppET-1 mRNA was significantly high compared to olodaterol alone, i.e. oxotremorine exerted still a significant stimulation effect, although smaller than that evoked by oxotremorine alone.

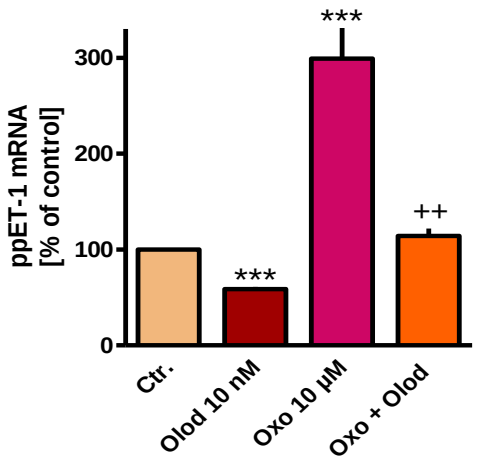


Figure 3-16: Effects of olodaterol (Oldo, 10 nM) and/or oxotremorine (Oxo, 10 µM) on expression of ppET-1 in MRC-5 human lung fibroblasts. After dissemination, cells were cultured for 24 hours in presence of 10% FCS followed by 24 hours in presence of 1% FCS and in absence of presence test drugs. Thereafter, total RNA was isolated, treated with DNase and used for quantitative real time PCR. *Height of columns:* ppET-1 (ΔCt over GAPDH) expressed as % of the Ctr. of the individual cell preparation, given are means + S.E.M. of n≥5 independent experiments. Significance of differences: ***P<0.01 vs Ctr.; $^{++}$P<0.01 vs Oxo or Oldo alone.

The effects of the muscarinic agonist, oxotremorine, and those of TGF-β appeared to be parallel in MRC-5 fibroblasts, as both mediated a strong stimulatory effect on ppET-1 mRNA expression. Therefore, possible interactions between cholinergic effects and the effects of TGF-β on ppET-1 mRNA expression were also studied. As illustrated in figure 3-17A, the stimulatory effect of the muscarinic agonist, oxotremorine was additive to that of TGF-β, but only when TGF-β was present in a low concentration of 0.1 ng/ml. In the presence of 0.3 ng/ml TGF-β, still not a maximally effective concentration, oxotremorine did not cause any further significant increase in the expression of ppET-1 mRNA.

Moreover, interaction experiments with the muscarinic antagonist tiotropium were performed to investigate whether non-neuronal acetylcholine synthesized by the fibroblasts might be involved in the action of TGF-β on the expression of ppET-1 mRNA. As shown in figure 3-17B, the muscarinic antagonist tiotropium did not affect TGF-β-induced up-regulation of ppET-1 mRNA expression.

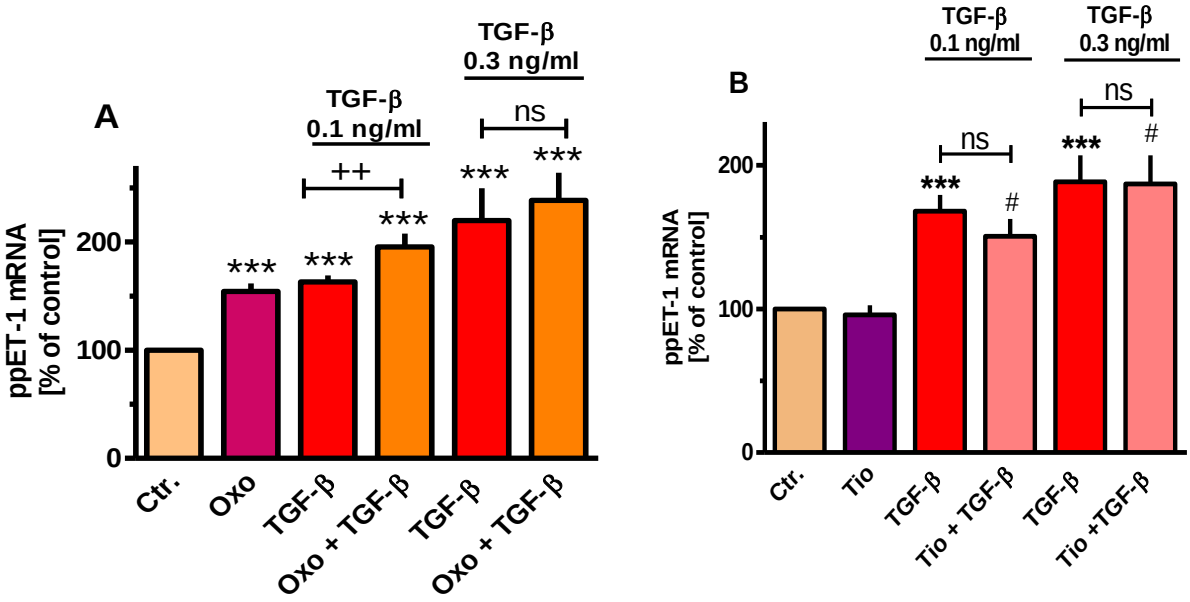


Figure 3-17: Effects of oxotremorine (Oxo, 10 µM, A) or tiotropium (Tio, 10 nM, B) and/or TGF-β (at the concentration indicated) on expression of ppET-1 in MRC-5 human lung fibroblasts. After dissemination, cells were cultured for 24 hours in presence of 10% FCS followed by 24 hours in presence of 1% FCS and in absence or presence of test drugs, Tio being present 30 min prior to Oxo. Thereafter, total RNA was isolated, treated with DNase and used for quantitative real time PCR. *Height of columns:* ppET-1 (ΔCt over GAPDH) expressed as % of the Ctr. of the individual cell preparation, given are means + S.E.M. n≥5 of independent experiments. Significance of differences: ***P<0.01 vs Ctr.; $^{++}$P<0.01 vs respective TGF-β alone; ns, not significant vs respective TGF-β alone; $^{\#}$P<0.001 vs Tio alone.

In order to test whether an autocrine/paracrine ET-1 release may contribute to the muscarinic stimulation of ppET-1 mRNA expression, interaction experiments with the non-selective ET-1 receptor antagonist bosentan was performed. As shown in figure 3-18, the stimulatory effect caused by 24 hours exposure of MRC-5 cells to 10 µM oxotremorine was largely attenuated by pre-treatment with bosentan (10 µM), suggesting that ET-1 actions via ET receptors are involved in oxotremorine-mediated up-regulation of ppET-1mRNA.

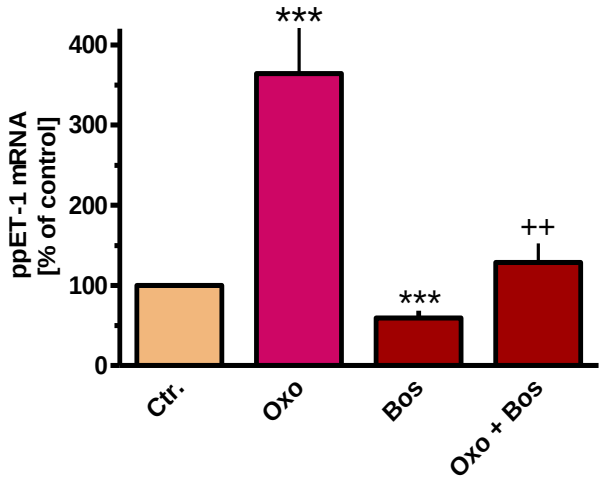
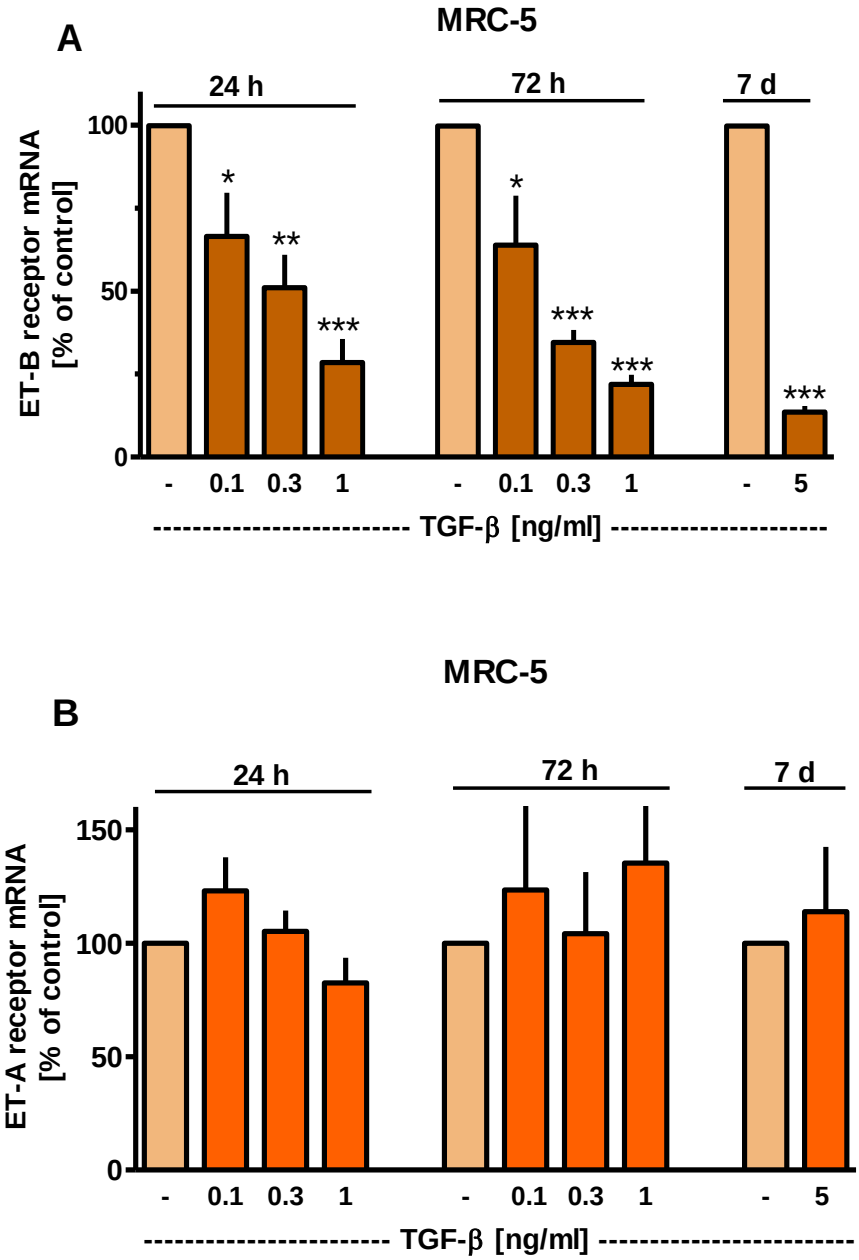


Figure 3-18: Effects of oxotremorine (Oxo, 10 µM) in presence or absence of bosentan (Bos, 10 µM) on expression of ppET-1 in MRC-5 human lung fibroblasts. After dissemination, cells were cultured for 24 hours in presence of 10% FCS followed by 24 hours in presence of 1% FCS and in absence or presence of test drugs, Bos being present 30 min prior to Oxo. Thereafter, total RNA was isolated, treated with DNase and used for quantitative real time PCR. *Height of columns:* ppET-1 (ΔCt over GAPDH) expressed as % of the Ctr. of the individual cell preparation, given are means + S.E.M. of n=7 independent experiments. Significance of differences: ***P<0.001 vs Ctr.; $^{++}$P<0.01 vs Oxo or Bos alone.

3.3. Regulation of ET receptor subtypes in human lung fibroblasts

3.3.1. Effects of TGF-β on ET receptor subtypes expression

The effects of TGF-β on ET receptor subtypes expression at transcriptional level were studied in human lung fibroblast cells using real time PCR. For this purpose, phLFb and MRC-5 cells were cultured up to one week in presence or absence of different concentrations of TGF-β (0.1, 0.3, 1 and 5 ng/ml). As demonstrated in figure 3-19A and 3-19C, in both MRC-5 and phLFb cells, TGF-β strongly down-regulated in a concentration dependent manner the expression of ET-B receptor mRNA. Contrary to this, the expression level of ET-A receptor was not significantly affected by TGF-β in MRC-5 cells (Fig. 3-19B) and only slightly reduced in phLFb cells after a prolonged 72 hour exposure (Fig. 3-19C).


A
MRC-5
24 h
72 h
7 d
ET-B receptor mRNA
[% of control]
100
50
0
*
**
***
*
***
***
-
0.1
0.3
1
-
0.1
0.3
1
-
5
TGF-β [ng/ml]
B
MRC-5
24 h
72 h
7 d
ET-A receptor mRNA
[% of control]
150
100
50
0
-
0.1
0.3
1
-
0.1
0.3
1
-
5
TGF-β [ng/ml]

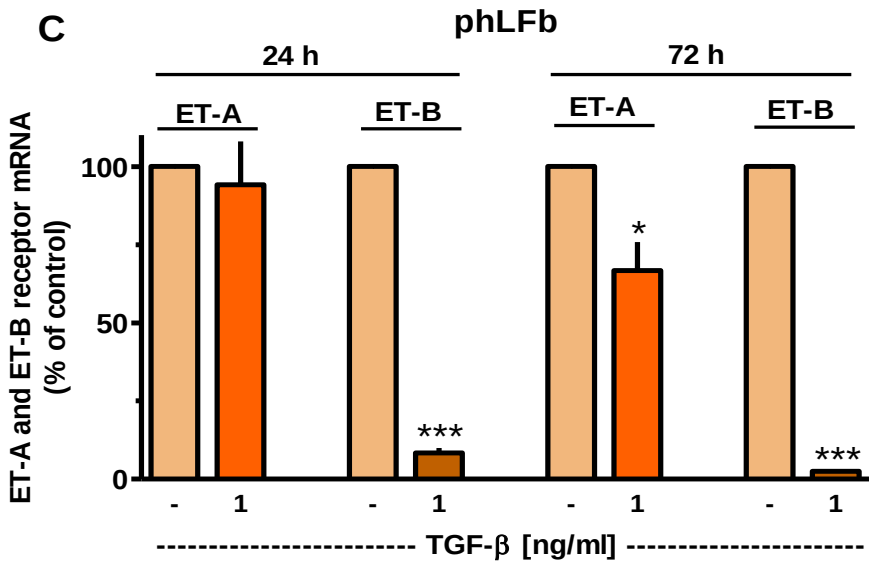


Figure 3-19: Effects of TGF-β on expression of ET-A and ET-B receptors in MRC-5 and primary (phLFb) human lung fibroblasts. After dissemination cells were cultured for 24 h in presence of 10% FCS followed by 24, 72 hours or 7 days in presence of 1% FCS and absence or presence of TGF-β at the concentrations indicated. Thereafter, total RNA was isolated, treated with DNase and used for quantitative real time PCR. *Height of columns:* ET-A or ET-B mRNA (ΔCt over GAPDH) expressed as % of the control of the individual cell preparation, given are means + S.E.M of n≥3 independent experiments. Significance of differences: ***P<0.001; **P<0.01; *P<0.05 vs respective Ctr..

3.4. Functional role of ET-1 and its receptors in human lung fibroblasts

The functional role of ET-1 and its receptor subtypes in specific cellular functions, including proliferation, collagen synthesis and modulation of phenotype in human lung fibroblasts were studied.

3.4.1. Effects of ET-1 and ET receptor subtype antagonists on proliferation

To evaluate whether fibroblast proliferation is regulated by endothelinergic mechanisms, the effects of ET-1 on proliferation were studied using [^{3}H]-thymidine incorporation assay. To establish an efficient protocol, different experimental conditions were examined. The conditions were checked for culture conditions and exposure time.

(A) In the first set of experiments, cells were seeded into 12-well dishes at a density of 4 x 10^4 cells per well and cultured for 24 hours in the presence of 10% FCS, followed by 18-24 hours under FCS-free conditions. After a 24-hour FCS free period,

increasing concentrations of ET-1 were added and present for 30 hours under FCS-free conditions, and [^{3}H]-thymidine (37 kBq) was present during the last 24 hours (i.e. it was added 6 hours after addition of ET-1). Under controlled conditions, ET-1 caused a trend of increased proliferation of MRC-5 fibroblasts, but it was minor, with an increase in [^{3}H]-thymidine incorporation, maximally by about 15% in MRC-5 fibroblasts (Fig. 3-20A). However, under these conditions the proliferative effect of ET-1 was substantially more pronounced in phLFb than in MRC-5 fibroblasts, with an increase in [^{3}H]-thymidine incorporation, maximally by about 74 ± 6% relative to the control levels (Fig. 3-20B).

(B) In a second set of experiments, cells were seeded into 12-well dishes at density of 5 x 10^4 cells per well and cultured for 5 hours in the presence of 10% FCS. Thereafter, cells were starved overnight in serum free medium, exposed subsequently for 30 hours to ET-1 at varying concentrations, and [^{3}H]-thymidine (37 kBq) was present during the last 24 hours. Under these conditions, ET-1 concentration-dependently increased [^{3}H]-thymidine incorporation in both cell types, phLFb and MRC-5 fibroblasts maximally by about 39 ± 6% and 56 ± 10% at 100 nM respectively (Fig. 3-20C).

(C) In the third protocol, with the intention of reducing the influence of a possible endogenous endothelinergic tone, cells were FCS-deprived and varying concentrations of ET-1 were applied from the onset of the test culture period, and cells were incubated for 30 hours with [^{3}H]-thymidine (37 kBq) present for the last 24 hours. Under these conditions, ET-1 strongly induced proliferation of phLFb and MRC-5 fibroblasts with an increase in [^{3}H]-thymidine incorporation by about 100% relative to the control levels (Fig. 3-20D). Noteworthy, for both cells types the maximum effect appears already reached at lower concentrations of ET-1 than under conditions described in above (Fig. 3-20 A-C).

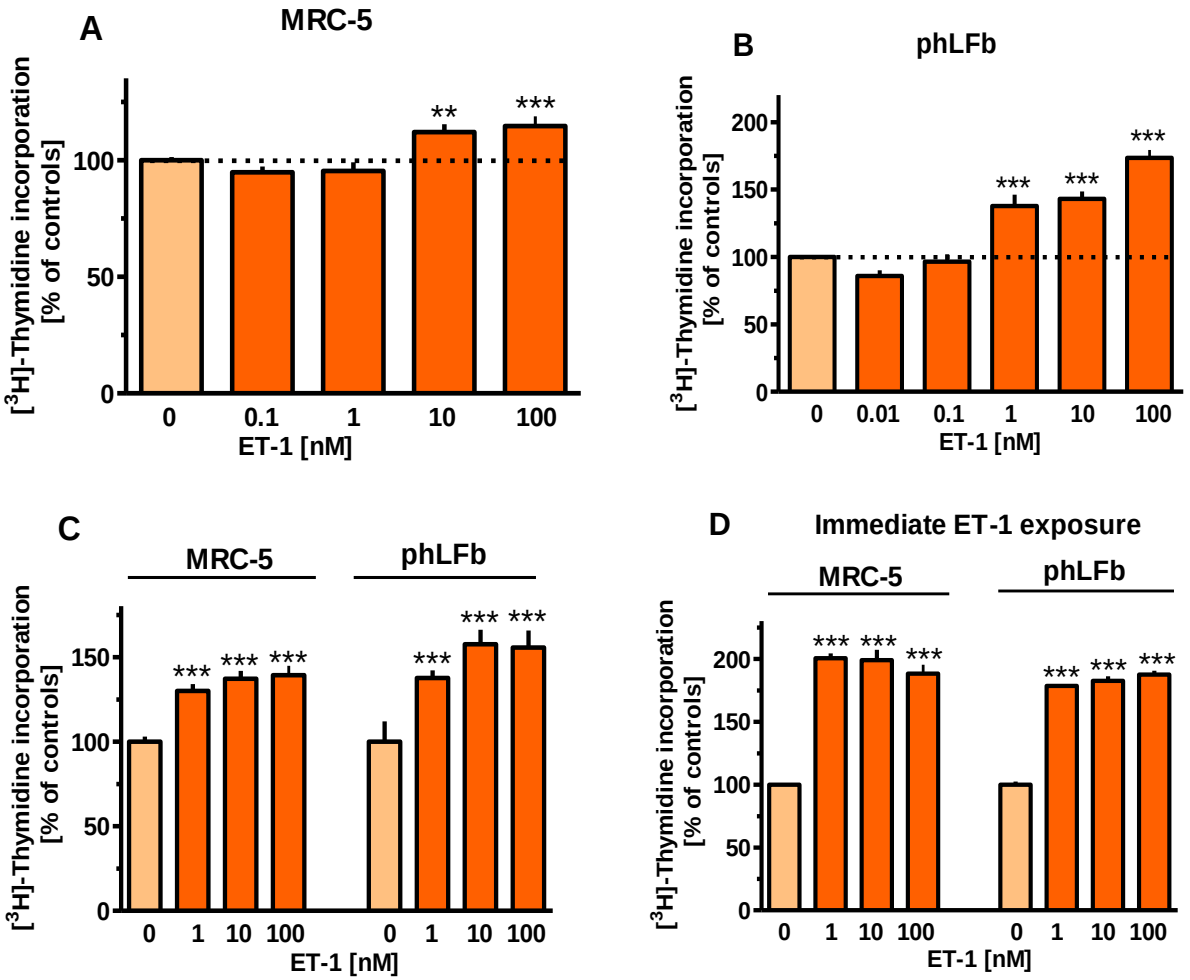


Figure 3-20: Effects of ET-1 on [^{3}H]-thymidine incorporation (A-D) in MRC-5 and primary (phLFb) human lung fibroblasts. **A** and **B**: 4 x10^4 cells were seeded in 12-well dishes and first cultured for 24 hours in presence of 10% FCS, followed by additional 24 hours under FCS-free conditions. After a 24 hours FCS-free period ET-1 was added as indicated for 30 hours. [^{3}H]-thymidine (37 kBq) was present for the last 24 hours (i.e. it was added 6 hours after addition of test drugs). **C:** 7.5 x 10^4 cells were seeded in 12-well dishes and first cultured for 5 hours in presence of 10% FCS. Thereafter, cells were starved overnight in serum free medium, stimulated subsequently for 30 hours with ET-1 and [^{3}H]-thymidine was present during the last 24 hours. **D:** 2 x 10^5 cells were seeded in 12-well dishes and cultured from the onset under FCS-free conditions for 30 hours in absence or presence of ET-1, and [^{3}H]-thymidine (37 kBq) was present for the last 24 hours. Cellular radioactivity expressed as % of the mean value of the controls of each cell preparation. Given are means + S.E.M of n≥9 independent experiments. Significance of differences: **P<0.01; ***P<0.001 vs respective controls.

In order to prove the specificity of the effect of ET-1 on which of the ET-receptor subtype mediates proliferation of human lung fibroblasts, interaction experiments with the selective ET-receptor antagonists, BQ-123 (ET-A) and BQ-788 (ET-B), as well as the non-selective ET receptor antagonist, bosentan were

performed. For this purpose, condition (A) was applied. As depicted in figure 3-21A, in MRC-5 fibroblasts, bosentan and ET-A antagonist BQ-123 concentration dependently reduced the basal [^{3}H]-thymidine incorporation, maximally by about 45% and 30%, respectively, whereas the ET-B antagonist BQ-788 had no significant effects. ET-1 alone caused only a minor increase in [^{3}H]-thymidine incorporation, maximally by about 15%. However, ET-1 was able to surmount in a concentration dependent manner the inhibitory effect of bosentan (Fig. 3-21B).

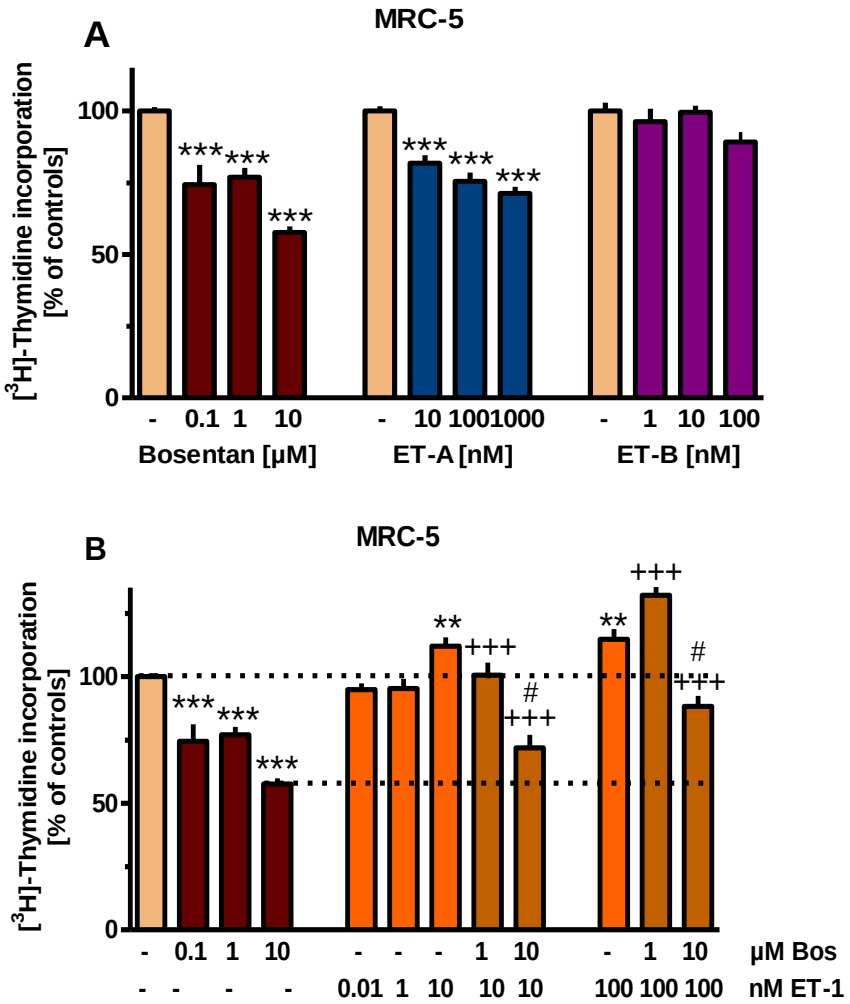
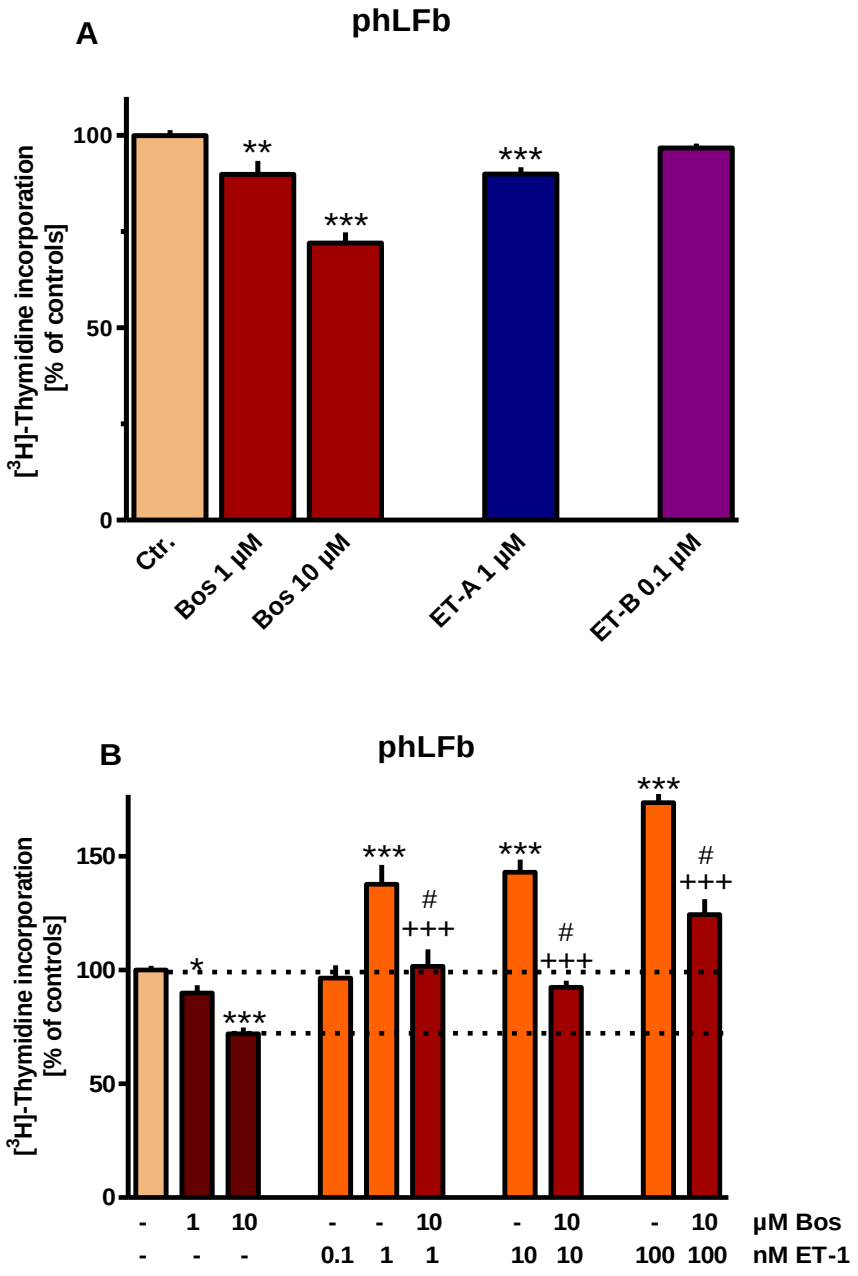


Figure 3-21: Effects of bosentan, the ET-A (BQ-123) and ET-B (BQ-788) receptor selective antagonist (A) and ET-1 in absence or presence of bosentan (Bos) on [^{3}H]-thymidine incorporation (B) in MRC-5 human lung fibroblasts. 4 x 10^4 cells were seeded in 12-well dishes and first cultured for 24 hours in presence of 10% FCS, followed by additional 24 hours under FCS-free conditions. After a 24 hours FCS-free period test drugs were added as indicated for 30 hours. [^{3}H]-thymidine (37 kBq) was present for the last 24 hours (i.e. it was added 6 hours after addition of test drugs). B: Bosentan was present 30 min before addition of ET-1, and [^{3}H]-thymidine during the last 24 hours. Cellular radioactivity expressed as % of the mean value of the controls of each cell preparation. Given are means + S.E.M of n≥9 experiments. Significance of differences: **P<0.01; ***P<0.001 vs respective controls; $^{+++}$P<0.001 vs respective value in presence of Bos alone; $^{\#}$P<0.001 vs respective value in absence of Bos.

Furthermore, in phLFb, under basal conditions, bosentan and BQ-123 caused also a reduction in [^{3}H]-thymidine incorporation, maximally by about 28% and 10% respectively. The maximum inhibitory effect bosentan and BQ-123 was smaller than that observed in MRC-5 cells, whereas, ET-B antagonist BQ-788 had no significant effects (Fig. 3-22A). Moreover, ET-1 was also able to surmount in a concentration dependent manner the inhibitory effect of bosentan and BQ-123 (Fig. 3-22B and 3-22C). BQ-788 did not significantly affect the ET-1-mediated increase in [^{3}H]-thymidine incorporation at concentrations up to 1 µM (data not shown).



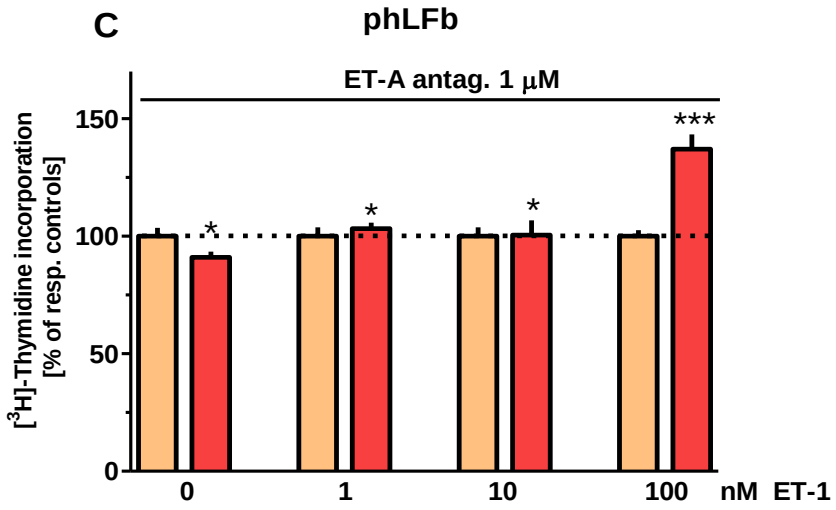


Figure 3-22: Effects of bosentan, the ET-A (BQ-123) and ET-B (BQ-788) receptor selective antagonist (A) and ET-1 in absence or presence of bosentan (Bos, B) or in absence or presence of ET-A (BQ-123, C) on [^{3}H]-thymidine incorporation in primary human lung fibroblasts. 4 x 10^4 cells were seeded in 12-well dishes and first cultured for 24 hours in presence of 10% FCS, followed by additional 24 hours under FCS-free conditions. After a 24 hours FCS-free period test drugs were added as indicated for 30 hours. [^{3}H]-thymidine (37 kBq) was present for the last 24 hours (i.e. it was added 6 hours after addition of test drugs). B: Bosentan was present 30 minute before addition of ET-1, and [^{3}H]-thymidine during the last 24 hours. Cellular radioactivity expressed as % of the mean value of the Ctr. of each cell preparation. Given are means + S.E.M of n≥9 independent experiments. Significance of differences: *P<0.05; **P<0.01; ***P<0.001 vs respective Ctr.; $^{+++}$P<0.001 vs respective value in presence of Bos alone; $^{\#}$P<0.001 vs respective value in absence of Bos.

3.4.2. Effect of ET-1 on cell numbers

Since DNA synthesis may occur without cell division, cell numbers were examined to verify that the increase in DNA synthesis induced by ET-1 in human lung fibroblast cells as assessed by [^{3}H]-thymidine incorporation assay indeed reflects an increase in cell proliferation. MRC-5 cells were cultured under FCS-free condition for 28 and 48 hours which are comparable with those conditions used in [^{3}H]-thymidine incorporation assay, where ET-1 exhibited strong effects. The number of cells under control conditions was 45000 ± 5300 (at 28 hours, n = 22) and 63500 ± 3850 (at 48 hours, n = 18, P < 0.05 vs. 24 hours). Exposure of the cells to ET-1 (10 nM) caused a marked increase in the number of MRC-5 cells by about 70% and 80% respectively (Fig. 3-23). In comparison, the addition of 1% FCS for 28 hours caused an increase in the number of cells by 70%. Furthermore, to verify that ET receptors

were also involved in this effect, interaction experiments with bosentan (10 µM) were performed. The cell count in the presence of bosentan (10 µM) alone tended to be reduced after 28 hours and was significantly reduced by 35% after 48 hours. In addition, bosentan prevented an ET-1-mediated increase in cell numbers.

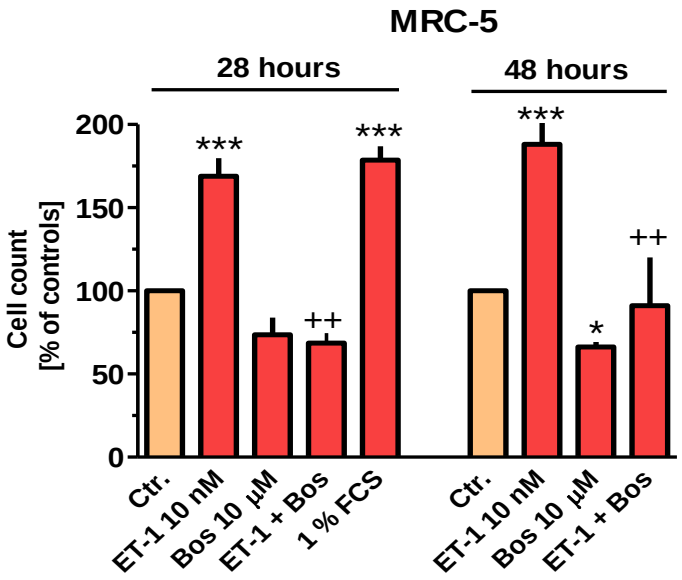


Figure 3-23: Effects of ET-1 in absence or presence of bosentan (Bos) on cell count in MRC-5 human lung fibroblasts. 7.5 x 10^4 cells were seeded in 12-well dishes and cultured from the onset under FCS-free conditions for 28 or 48 hours in absence or presence of test substance (include 1% FCS). Cell count expressed as % of the mean value of the Ctr. of each cell preparation. Given are means + SEM of *n* = 18–22 independent experiments. Significance of differences: **P* < 0.05; ****P* < 0.001 versus respective Ctr.; ^{++}P < 0.01; versus respective value in absence of Bos.

3.4.3. Role of the Rho-kinase in ET-1-induced proliferation

In order to investigate whether the Rho kinase might be involved in the endothelinergic stimulation of proliferation of MRC-5 human lung fibroblasts, interaction experiments with Rho kinase inhibitor Y-27632 were performed. For this purpose, cells condition (C) was applied. As shown in figure 3-24, ET-1 significantly enhanced [^{3}H]-thymidine incorporation, while the Rho-kinase inhibitor Y-27632 (3 µM) reduced [^{3}H]-thymidine incorporation by about 40 %. However, the Rho-kinase inhibitor Y-27632 did not inhibit ET-1 induced stimulation of [^{3}H]-thymidine incorporation (Fig. 3-24).

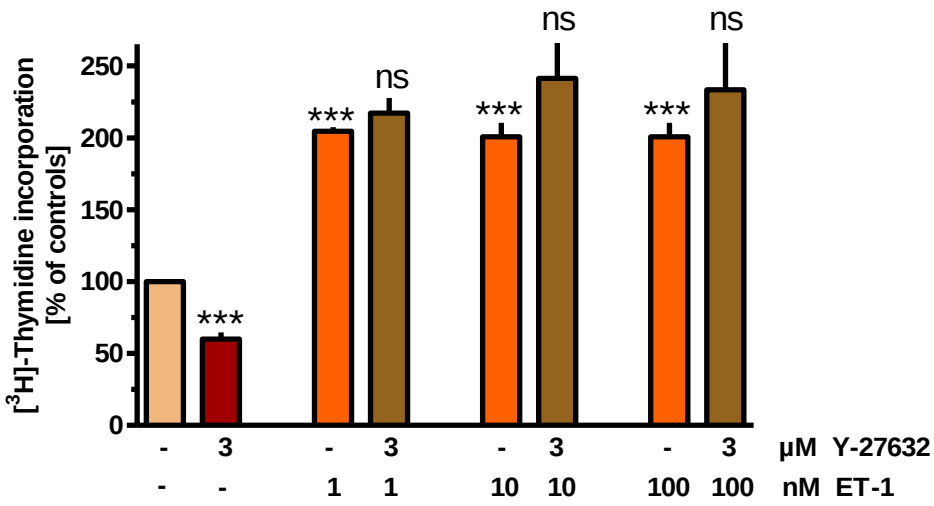


Figure 3-24: Effects of the Rho-kinase inhibitor Y-27632 on ET-1 mediated increase in [^{3}H]-thymidine incorporation in MRC-5 human lung fibroblast proliferation. 2 x10^5 cells were seeded in 12 well dishes in the absence of FCS and cultured for 30 hours with or without test drugs as indicated. Y-27632 was present 30 minutes before the addition of ET-1, and [^{3}H]-thymidine (37 kBq) was applied for the last 24 hours (i.e. it was added 6 hours after addition of ET-1). Cellular radioactivity expressed as % of the mean value of the Ctr. of each cell preparation. Given are means ± SEM of n=6 independent experiments. Significance of differences: ***P<0.001 vs respective Ctr.; ns, not significant vs respective Y-27632 alone.

3.4.4. Effects of ET-1 and ET receptor antagonist on myofibroblast differentiation

To verify whether ET-1 and ET receptors were involved in the differentiation of MRC-5 fibroblast to myofibroblast, the effect of ET-1 and the non selective ET receptor antagonist, bosentan on the expression of smooth muscle actin protein (α-SMA), a marker of myofibroblast differentiation was examined by Western blot analysis. MRC-5 cells cultured under standard conditions showed expression of α-SMA (Fig. 3-25A). Exposure of the cells to bosentan (10 µM), for a 24 hour period, caused a clear reduction in α-SMA protein levels by about 50%, whereas ET-1 10 nM (Fig. 3-25B) or 100 nM (data not shown) induced only a minor increase by about 25%.

Furthermore, exposure of the cells for 24 hours to 0.3 ng/ml TGF-β, caused an increase in α-SMA protein expression by about 120%, whereas bosentan (10 µM) had no significant effect on TGF-β-mediated increase in α-SMA protein expression (Fig. 3-25B).

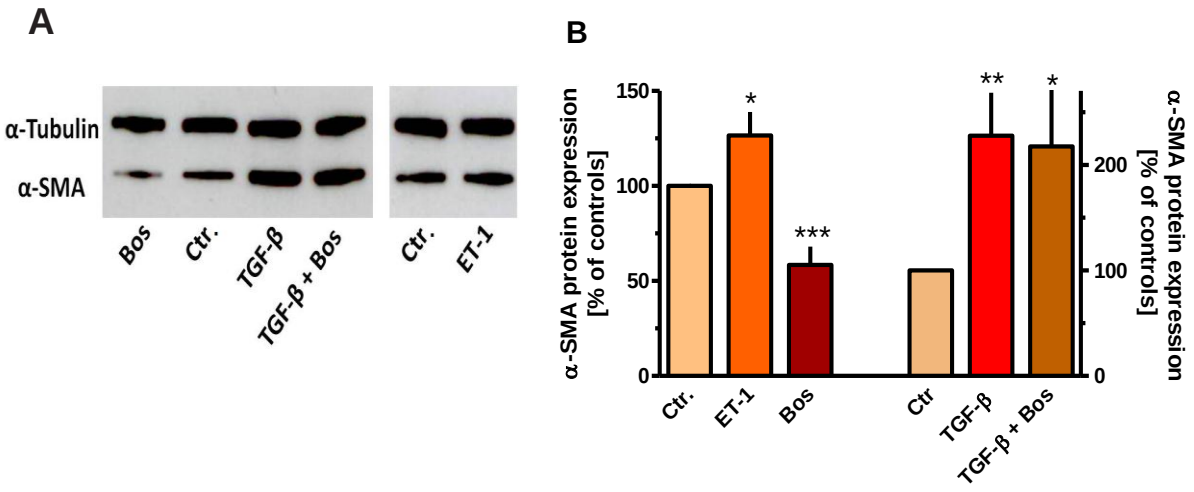


Figure 3-25: Effects of the ET-1 (10 nM), TGF-β (0.3 ng/ml) and/or bosentan (Bos, 10 µM) on α-smooth muscle actin (α-SMA) expression in MRC-5 human lung fibroblasts. 3 x 10^5 cells were seeded in 35 mm dishes and cultured for 24 hours in presence of 10% FCS. After a 24 hours FCS-free period, cells were cultured for additional 24 hours in FCS-free 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 Ctr.. Given are means + S.E.M of n≥7 independent experiments. Significance of differences: *P<0.05, **P<0.01, ***P<0.001 vs Ctr..

3.4.5. Role of ET-1 and its receptors in the regulation of collagen synthesis

To investigate whether ET-1 may contribute to fibrotic responses through the enhancement of collagen synthesis, the effect of ET-1 on collagen synthesis, as determined by [^{3}H]-proline incorporation was investigated. In MRC-5 fibroblasts, under controlled conditions (i.e., absence of FCS and any test drugs) [^{3}H]-proline incorporation into cellular proteins during 24 hours incubation amounted to 30.557 ± 2.163 d.p.m. (disintegration per minute) (n=45). As shown in figure 3-26A, ET-1 (1-100 nM) caused a concentration-dependent increase in [^{3}H]-proline incorporation, maximally by about 85% at 10 nM (EC_{50}: 0.7 nM). Therefore, in further experiments ET-1 at a concentration of 10 nM was used.

Additionally, to confirm the stimulatory effect of ET-1 on collagen synthesis in human lung fibroblasts, [^{3}H]-proline incorporation experiments were performed with primary human lung fibroblasts (phLFb). As demonstrated in figure 3-26B, [^{3}H]-proline incorporation was increased by ET-1 in a concentration dependant-manner, comparable to that observed in MRC-5 fibroblasts.

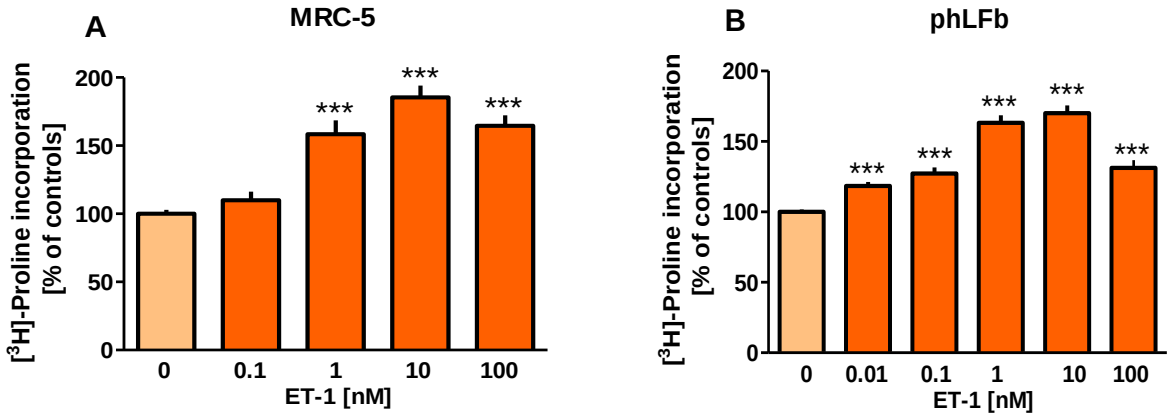


Figure 3-26: Effects of ET-1 on [^{3}H] proline incorporation in MRC-5 (A) and primary human lung fibroblasts (phLFb) (B). 10^5 cells were seeded in 12-well dishes and first cultured for 24 h in presence of 10% FCS, followed by additional 24 hours under FCS-free conditions. Thereafter, [^{3}H]-proline (37 kBq) was added alone or in combination with test drugs and cells were cultured for further 24 hours. Radioactivity incorporated in cellular and extracellular protein was determined. Data expressed as % of the mean value of the controls of each cell preparation. Given are means + S.E.M of n≥9 independent experiments. Significance of differences: ***P<0.001 vs controls (absence of drugs).

After investigating that ET-1 stimulates collagen synthesis in both MRC-5 fibroblasts and phlFb, it was important to further investigate which ET receptor subtype mediates this effect. To address this issue, interaction experiments with the selective ET-receptor antagonists, BQ-123 (ET-A) and BQ-788 (ET-B) as well as the non-selective ET-receptor antagonist, bosentan were performed. As shown in figure 3-27A, in confirmation of preceding observations (Fig. 3-26A) ET-1 enhanced the [^{3}H]-proline incorporation in MRC-5 cells in a concentration dependent manner, while bosentan and BQ-123 caused a reduction in [^{3}H]-proline incorporation by about 20%. BQ-788 had no significant effect on basal [^{3}H]-proline incorporation (Fig. 3-27A). The stimulatory effect of ET-1 on the collagen synthesis was effectively antagonized by pre-treatment of the cells with bosentan and BQ-123. In contrast, blocking ET-B with BQ-788 had no effect (Fig. 3-27A).

Furthermore, in phLFb, none of the ET receptor antagonists significantly affected the basal levels of [^{3}H]-proline incorporation (Fig. 3-27B). However, like in MRC-5 cells, the effect of ET-1 was effectively, but in a surmountable manner, antagonized by bosentan and BQ-123, whereas BQ-788, did not significantly affect the effect of ET-1 response (Fig. 3-27B). In both cell types the antagonistic effect of bosentan and BQ-123 was surmountable, suggestion a competitive interaction between ET-1 and the antagonist.

Moreover, there is evidence that blockade of ET-A receptors could unmask an ET-B receptor-mediated effect in MRC-5 fibroblasts as determined by dynamic mass redistribution measurement (Ahmedat *et al.*, 2013). Therefore, BQ-788 was also studied in presence of BQ-123 either alone or in combination with 10 or 100 nM ET-1, but again failed to affect [^{3}H]-proline incorporation (data not show, each n=6-9).

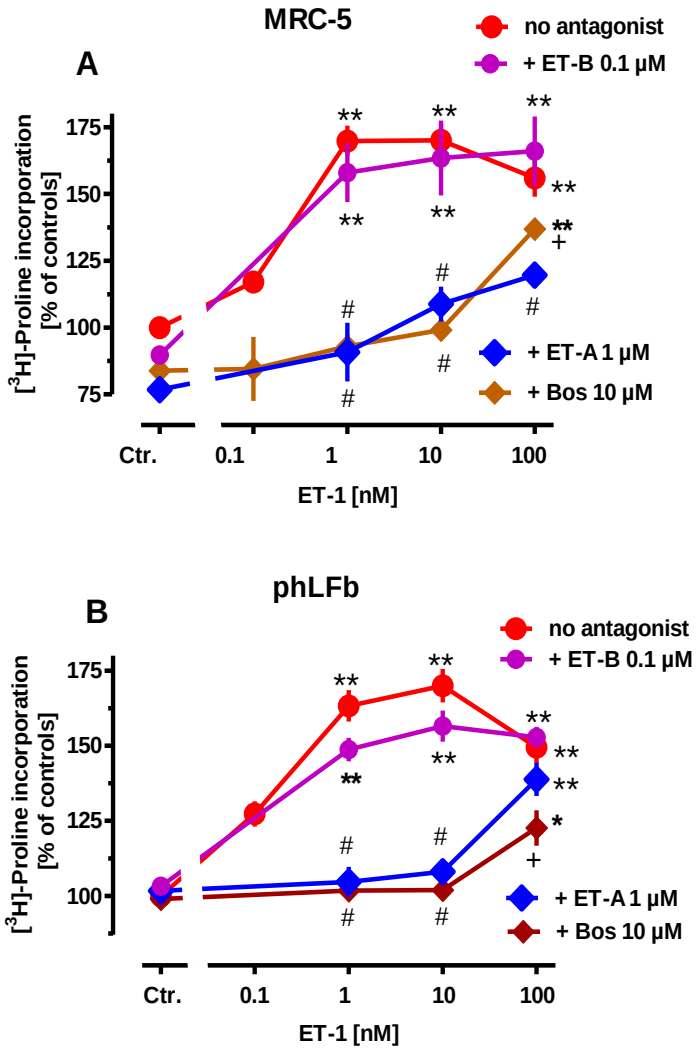


Figure 3-27: Concentration-dependent effects of ET-1 in absence and presence of bosentan (Bos) or the ET-A (BQ-123) or ET-B (BQ7-88) receptor selective antagonist on [^{3}H]-proline incorporation in MRC-5 (A) phLFb (B). 10^5 cells were seeded in 12-well dishes and first cultured for 24 hours in presence of 10% FCS, followed by additional 24 hours under FCS-free conditions. Thereafter, [^{3}H]-proline (37 kBq) was added alone or in combination with test drugs and cells were cultured for further 24 hours. Bos (10 µM) or BQ-123 (1 µM) or BQ-788 (100 nM) were present 30 minute prior to exposure to ET-1. Radioactivity incorporated in cellular and extracellular protein was determined. Data expressed as % of the mean value of the controls of each cell preparation. Given are means + S.E.M of n≥9 independent experiments. Significance of differences: *P<0.05, **P<0.01, ***P<0.001 vs Ctr. (absence of drugs); $^+$P<0.05; $^\#$P<0.01 vs respective value in absence of antagonists.

In addition, the stimulatory effects of ET-1 on collagen synthesis was further evidenced by directly measuring of collagen, using the QuickZyme collagen assay that is based on specific binding of the dye Sirius Red to collagen. The amount of collagen detected in control cultures was 5.1 ± 0.7 µg (n=5). Exposure of MRC-5 cells to ET-1 for 24 hours caused in a concentration-dependent manner, a substantial increase of the amount of collagen, maximally by 175% at 10 nM (Fig. 3-28). Interestingly, there was no further increment in the collagen synthesis by increasing the concentration above 10 nM of ET-1. The results observed in two different assays ([^{3}H]-proline incorporation assay and QuickZyme collagen assay) were comparable and consistent.

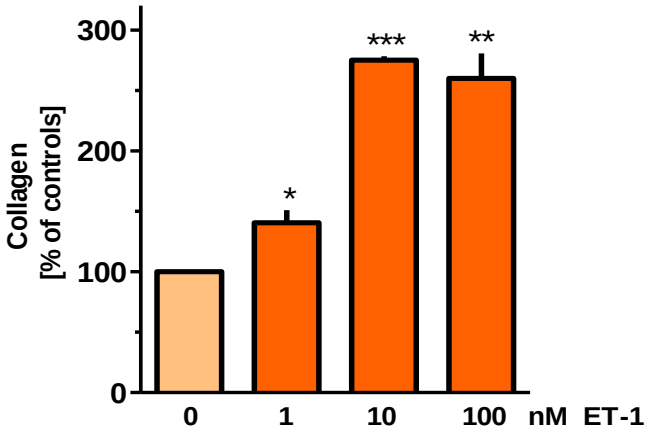


Figure 3-28: Effect of ET-1 on collagen synthesis by MRC-5 human lung fibroblasts. 10^5 cells were seeded in 24-well dishes and first cultured for 24 hours in presence of 10% FCS, followed by additional 24 hours under FCS-free conditions. Thereafter, cells were cultured for further 24 hours in absence or present of different concentrations of ET-1. Collagen content of cells and extracellular matrix was determined by the QuickZyme collagen assay. Data expressed as % of the mean value of the controls of each cell preparation. Given are means + S.E.M of n= 5 experiments. Significance of differences: *P<0.05; **P<0.01 and ***P<0.001 vs controls (absence of drugs).

3.4.6. Effect of ET-1 on COL I-α1 mRNA expression

To test whether an enhanced collagen gene transcription contributes to the ET-1-induces increase in the collagen synthesis in human lung fibroblasts, the effect of ET-1 on the expression of COL I-α1 mRNA, a major collagen type in lung fibroblasts, was examined. As shown in figure 3-29, exposure of MRC-5 cells to 10 nM and 100 nM ET-1 for 24 hours, caused an increase in the expression levels of COL I-α1 mRNA, maximally by about 60%.

Furthermore, the increase in COL I-α1 mRNA expression induced by 10 nM and 100 nM ET-1 was prevented by pre-treatment with the non-selective ET-receptor antagonist bosentan (10 µM), which alone had no effect on the expression of COL I-α1 mRNA (Fig 3-29).

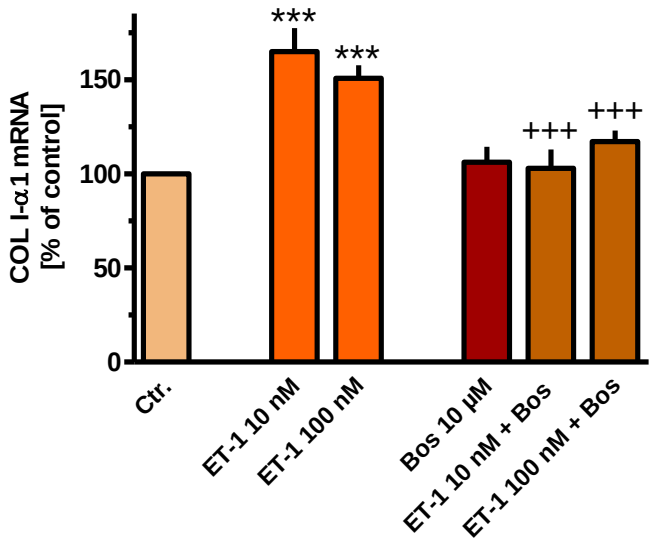


Figure 3-29: Effect of ET-1 in absence or presence of bosentan (Bos) on COL I-α1 mRNA expression in MRC-5 human lung fibroblasts. 3 x 10^5 cells were seeded in 6-well dishes and first cultured for 24 hours in presence of 10% FCS, followed by additional 24 hours under FCS-free conditions in absence or presence of test substances at the concentrations given. Thereafter, total RNA was isolated, treated with DNase and used for quantitative real time PCR. *Height of columns*: collagen I-α1 mRNA (ΔCt over GAPDH) expressed as % of the Ctr. of the individual cell preparation, given are means + S.E.M of n≥6 independent experiments. Significance of differences: ***P<0.01 vs Ctr., $^{+++}$P<0.001 vs respective value in absence of Bos.

3.4.7. Effects of ET receptor subtype antagonists on TGF-β-mediated collagen synthesis stimulation

As the preceding results showed that TGF-β enhanced ppET-1 expression and ET-1 release in MRC-5 fibroblasts, it was further examined whether the stimulatory effect of TGF-β on collagen synthesis involves ET-1. Therefore interaction experiments with the selective ET-A (BQ-123) or ET-B (BQ-788) receptor antagonists, as well as the non selective ET receptor antagonist bosentan were performed. As shown in figure 3-30A, TGF-β in the relatively low concentrations of 0.1, 0.3 and 1 ng/ml exhibited strong stimulatory effect on [^{3}H]-proline incorporation, maximally by about 80% at 0.3 ng/ml. Bosentan (10 µM) and BQ-123 (1 µM) under

control conditions caused (like in preceding experiments, Fig. 3-27A) a reduction in [^{3}H]-proline incorporation by about 20% (Fig. 3-30B-C), whereas BQ-788 (100 nM) had no effect (Fig. 3-30C). However, in presence of bosentan or BQ-123, TGF-β still induced significant increases in [^{3}H]-proline incorporation, but its effect was significantly attenuated, interestingly, compared to control conditions (Fig. 3-30B and C) a significantly large fraction was sensitive to ET receptor blockade by bosentan (10 µM) or BQ-123 (1 µM). BQ-788 (100 nM) failed to inhibit the TGF-β-mediated increase in [^{3}H] proline incorporation (Fig. 3-30D).

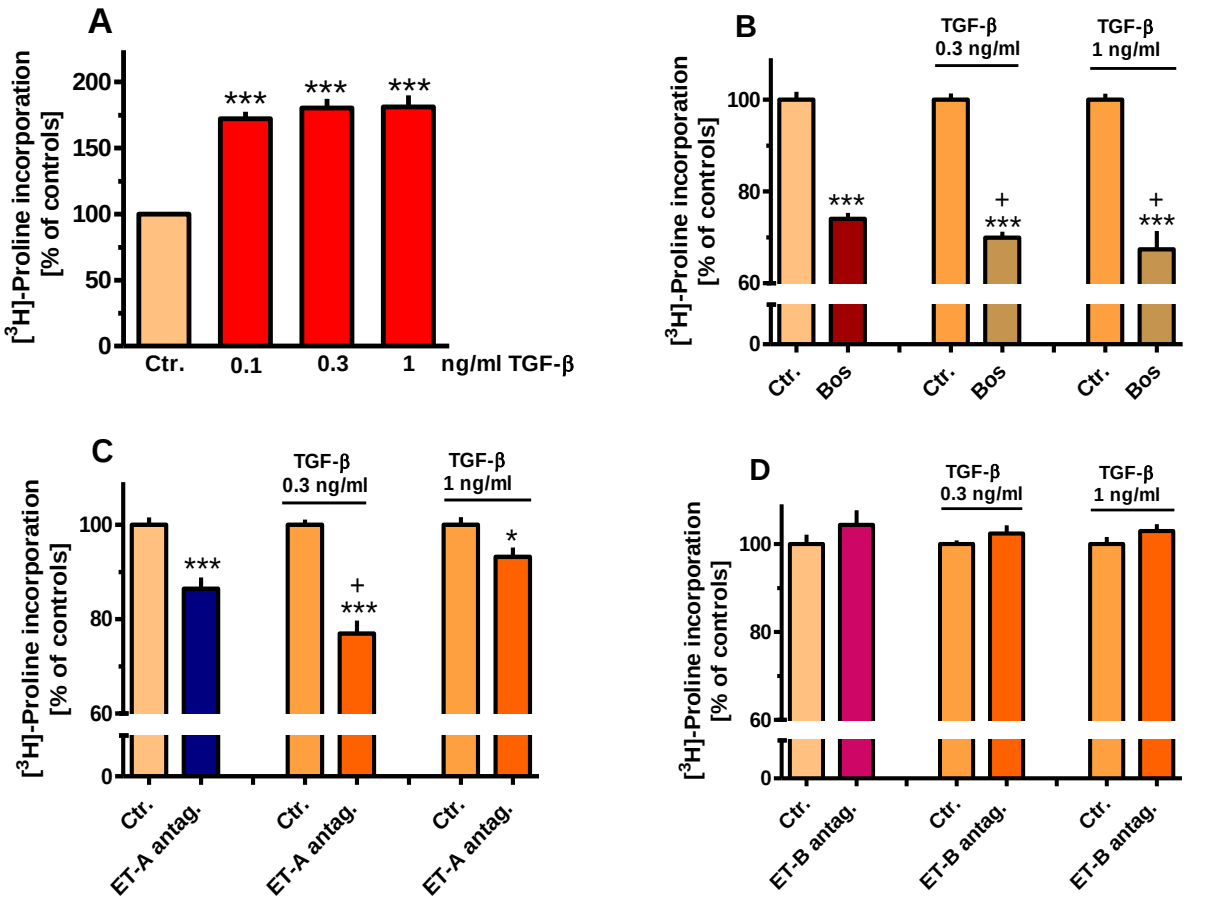


Figure 3-30: Effects of TGF-β (0.1, 0.3 and 1 ng/ml) (A) and of bosentan (Bos, 10 µM) (B) or the ET-A (BQ123, 1 µM) (C) or the ET-B (BQ788, 100 nM) (D) receptor selective antagonist in absence and presence of 0.3 and 1 ng/ml TGF-β on [^{3}H]-proline incorporation in MRC-5. 10^5 cells were seeded in 12-well dishes and first cultured for 24 h in presence of 10% FCS, followed by additional 24 hours under FCS-free conditions. Thereafter, [^{3}H]-proline (37 kBq) was added alone or in combination with test drugs and cells were cultured for further 24 hours. Radioactivity incorporated in cellular and extracellular protein was determined and expressed as % of the mean value of the respective Ctr. (in absence or presence of TGF-β) of each cell preparation. Given are means + S.E.M of n≥8 independent experiments. Significance of differences: *P<0.05, ***P<0.001 vs respective Ctr.; $^+$P<0.05 vs respective value in absence of TGF-β.

3.4.8. Effect of ET receptor antagonist on muscarinic agonist-mediated collagen synthesis stimulation

As the preceding results showed that the muscarinic agonist oxotremorine increased the ppET-1 mRNA expression, it was further tested whether ET-1 up-regulated by oxotremorine might contribute to the pro-fibrotic effects of this mediator. Therefore, the effects of the non-selective ET-1 receptor antagonist bosentan on the stimulatory effect of oxotremorine on collagen synthesis were studied. Under control conditions, oxotremorine (10 µM) caused an increase in [^{3}H]-proline incorporation by about 50%, whereas bosentan slightly, but significantly, reduced the basal [^{3}H]-proline incorporation by about 20%, and attenuated the stimulatory effect of oxotremorine on [^{3}H]-proline incorporation (Fig 3-31A). In presence of bosentan, oxotremorine induced still a significant increase in [^{3}H]-proline incorporation, but its effect was smaller. In other words, in presence of oxotremorine, bosentan showed significantly stronger inhibitory effects (Fig. 3-31B.).

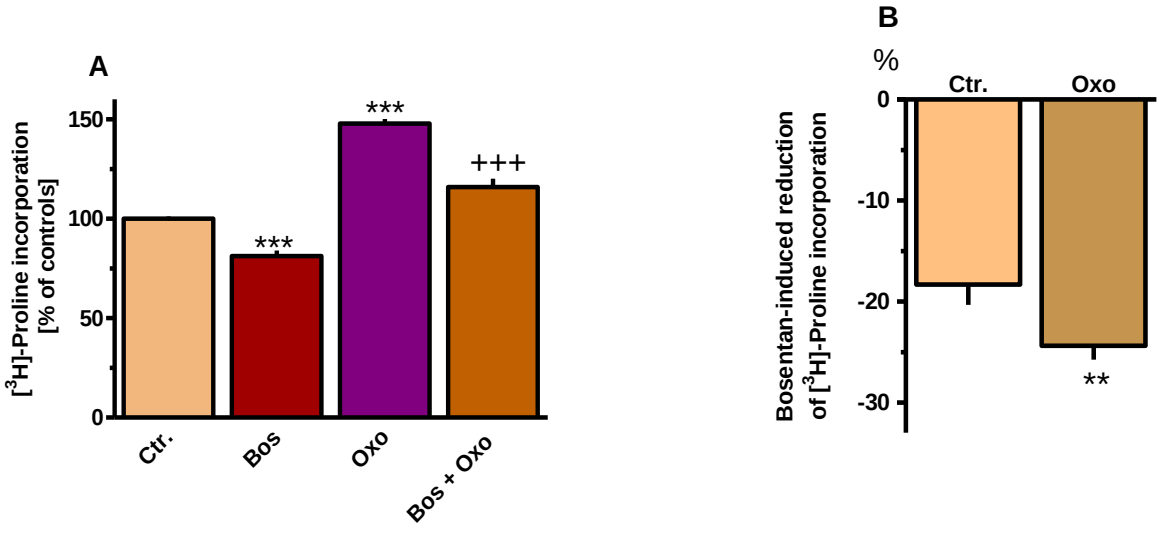


Figure 3-31: Effects of oxotremorine (Oxo, 10 µM) in absence and presence of bosentan (Bos, 10 µM) on [^{3}H]-proline incorporation in MRC-5 (A). 10^5 cells were seeded in 12-well dishes and first cultured for 24 h in presence of 10% FCS, followed by additional 24 hours under FCS-free conditions. Thereafter, [^{3}H]-proline (37 kBq) was added alone or in combination with test drugs and cells were cultured for further 24 hours. Bos was present 30 minute prior to the exposure to Oxo. Radioactivity incorporated in cellular and extracellular protein was determined and expressed as % of the mean value of the respective Ctr. (in absence of test substance, A) or the respective experiments in absence of bosentan (B) of each cell preparation. Given are means + S.E.M n≥9 of independent experiments. Significance of differences: **P<0.01, ***P<0.001 vs Ctr.; $^{+++}$P<0.001 vs respective value in presence of Bos or Oxo alone.

Additionally, possible interactions between cholinergic effects and the effects of TGF-β on collagen synthesis in MRC-5 fibroblasts were also studied. As shown in figure 3-32, the stimulatory effect of oxotremorine was additive to that of TGF-β, but only when TGF-β was present in a relatively low concentration of 0.1 ng/ml. In presence of 0.3 ng/ml TGF-β, still not a maximally effective concentration, oxotremorine did no longer cause a significant further increase in [^{3}H]-proline incorporation. The additive effect of TGF-β along with oxotremorine was consistent to that observed in the ET-1 expression (compare Figs. 3-15A and 3-32).

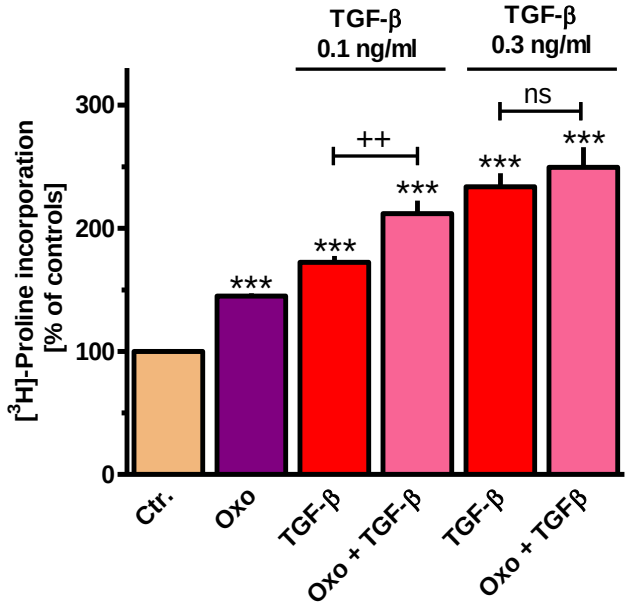
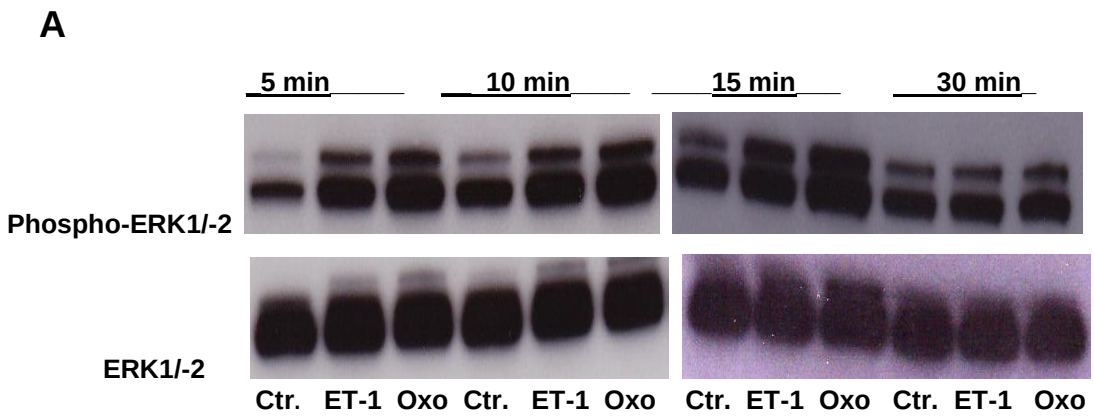


Figure 3-32: Effects of oxotremorine (Oxo, 10 µM) and/or TGF-β (0.1 or 0.3 ng/ml as indicated) on [^{3}H]-proline incorporation in MRC-5. 10^5 cells were seeded in 12-well dishes and first cultured for 24 hours in presence of 10% FCS, followed by additional 24 hours under FCS-free conditions. Thereafter, [^{3}H]-proline (37 kBq) was added alone or in combination with test drugs and cells were cultured for further 24 hours. Radioactivity incorporated in cellular and extracellular protein was determined and expressed as % of the mean value of the respective controls (in absence of test substance) or the respective experiments in absence of Oxo of each cell preparation. Given are means + S.E.M of n=9 independent experiments. Significance of differences: ***P<0.001 vs Ctr.; $^{++}$P<0.01 vs respective value in absence of Oxo; ns, not significant.

3.4.9. Effects of ET-1 and ET receptor subtype antagonists on ERK1/-2 phosphorylation

As previous studies in our laboratory demonstrated that the muscarinic receptor induced stimulation of proliferation and collagen synthesis in human lung fibroblasts was mediated by activation of the ERK MAP kinase pathway (Matthiesen *et al.*, 2007; Haag *et al.*, 2008a) a possible role of the MAPK pathway in ET-1 induced effects was analysed. In this regard, Western blot analysis was performed to examine whether ERK1/-2 MAPK cascade is activated by ET-1. Data shown in figure 3-33 indicate that ET-1, similar to the muscarinic agonist oxotremorine, induced a strong and rapid but transient activation of ERK1/-2. There was a rapid increase of phosphorylated ERK1/-2 in cells exposed to 10 and 100 nM of ET-1 maximally already within 5 minutes by 384 % and 409%, respectively. The maximum effect of ET-1 was somewhat larger than that of oxotremorine (10 µM, also a maximally effective concentration, Matthiesen *et al.,* 2007; Haag *et al.,* 2008a). The effect of ET-1 on ERK1/-2 rapidly declined, and phosphorylated ERK1/-2 returned to baseline levels within 30 minutes after ET-1 exposure. Therefore, in subsequent experiments, a five minute treatment with 10 nM ET-1 was used. Total amount of ERK1/-2 was not affected by ET-1.



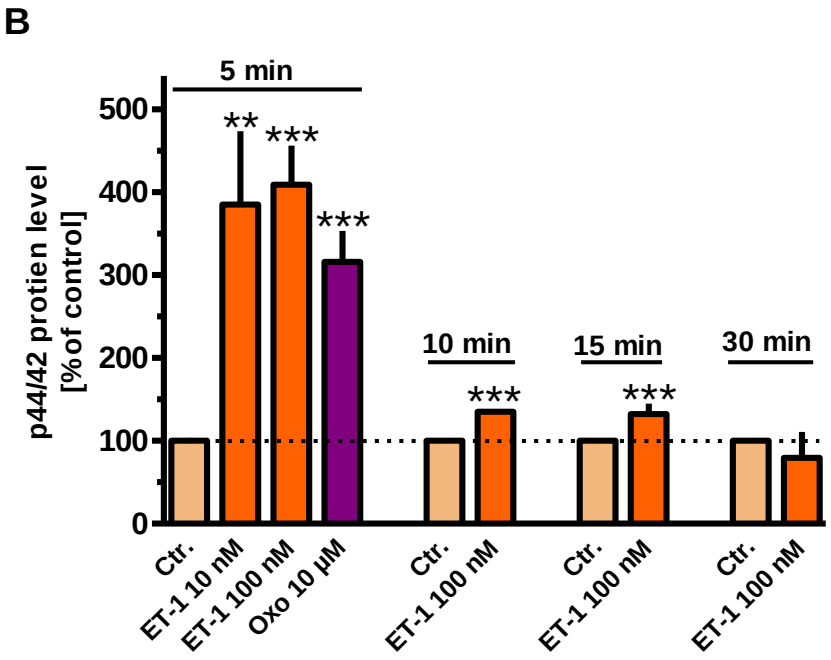


Figure 3-33: Western blot analysis of phospho-p42/44 (ERK1/-2) MAPK in MRC-5 human lung fibroblasts. Cells were cultured in 55 mm dishes to nearly confluence, serum-starved for 24 hours and exposed to 10 or 100 nM ET-1, 10 µM oxotremorine (Oxo), or vehicle (Ctr.) for 5–30 minute (as indicated). In controls, cells were treated with solvent at the respective time points (i.e. 5, 10, 15, and 30 min before cell lysis, respectively). Cell lysates were prepared and 20 µg were separated in 4-12% acrylamide Tris–glycine gels, immobilized to PVDF membranes and detected by antibodies specific for phosphorylated and unphosphorylated ERK. (A) Samples of original Western blots. (B) Densitometrical quantification of the p44/42 bands was performed and values (arbitrary units, normalized over unphosphorylated ERK) expressed as % of the respective Ctr.. Given are means + S.E.M of n=3–14 independent Western blots. Significance of differences: **P<0.01, ***P<0.001 vs respective Ctr..

Furthermore, in order to identify the ET receptor involved, interaction experiments with selective ET receptor antagonists BQ-123 (ET-A) and BQ-788 (ET-B) were performed. These compounds were added to MRC-5 cells for 30 minute prior to the ET-1 treatment and the solvent of the antagonists were also added to all samples 30 minute prior to the addition of ET-1 to keep all samples at the same experimental conditions. As shown in figure 3-34, BQ-123 caused a reduction in the basal phospho-p42/44 levels by about 30%. Moreover, the increase in phosphorylated ERK1/-2 MAPK induced by 10 and 100 nM ET-1 was significantly inhibited by 1 µM BQ-123 (by about 60% and 72% respectively with respect to presence the of ET-1), whereas BQ-788 (100 nM) showed no effects, neither in absence nor presence of ET-1. It should be mentioned that the solvent DMSO alone

(in the concentration present with BQ-788) significantly attenuated the stimulatory effect of ET-1. Therefore, the data in figure 3-34 show effects in relation to respective controls in absence or presence of the solvent.

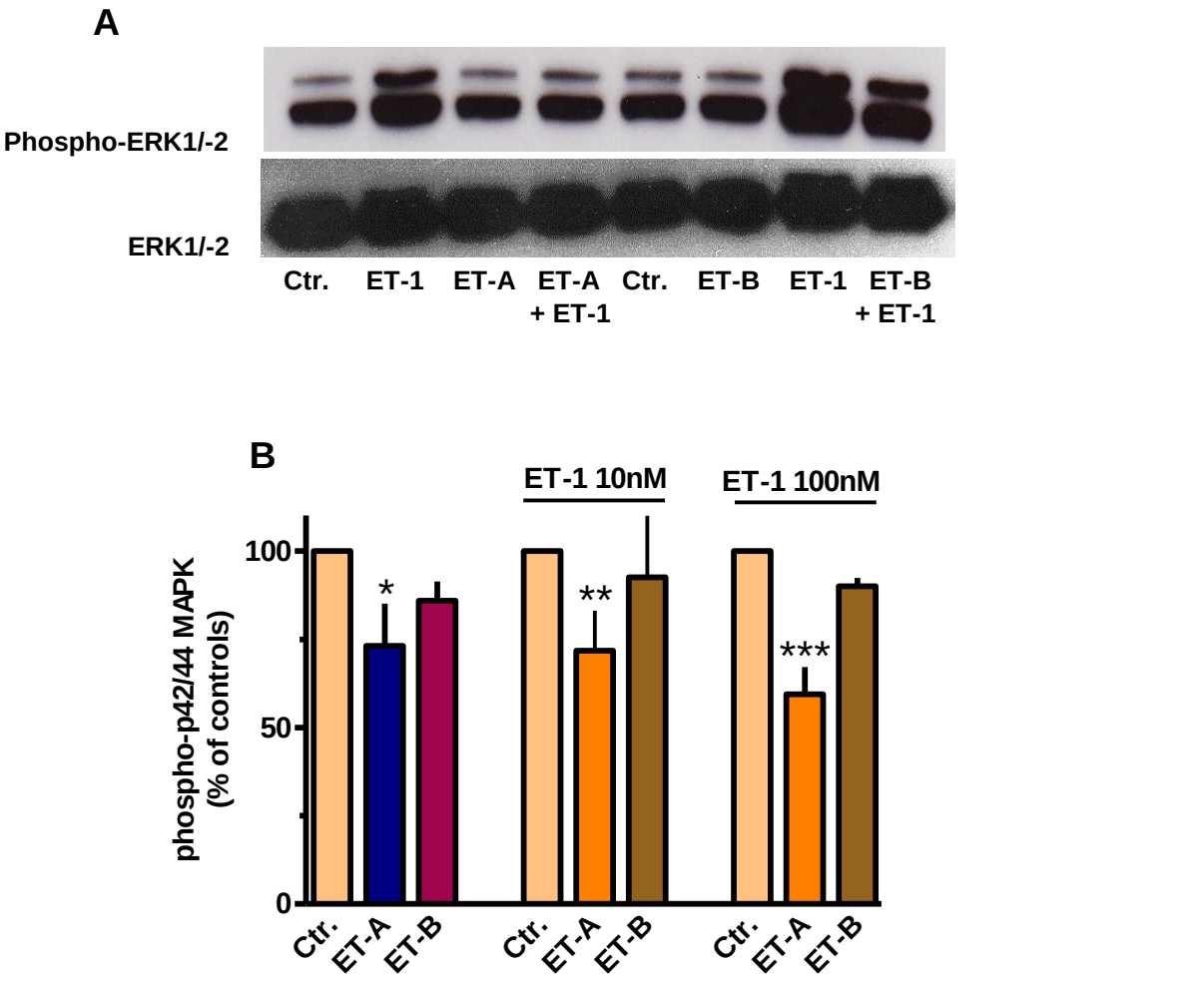


Figure 3-34: Western blot analysis of p42/44 (ERK1/-2) MAPK in MRC-5 human lung fibroblasts. Cells were cultured in 55 mm dishes to nearly confluence, serum-starved for 24 h and exposed to 10 or 100 nM ET-1, or vehicle (Ctr.) for 5–30 min (as indicated). The ET-A (BQ-123, 1 µM) or ET-B (BQ-788, 100 nM) receptor antagonist was present 30 min prior to exposure to ET-1. In controls, cells were treated with solvent at the respective time points (i.e. 5 min and additionally 30 min before cell lysis, respectively). Cell lysates were prepared and 20 µg were separated in 4-12% acrylamide Tris–glycine gels, immobilized to PVDF membranes and detected by antibodies specific for phosphorylated and unphosphorylated ERK. (A) Samples of original Western blots. (B) Densitometrical quantification of the p44/42 bands was performed and values (arbitrary units, normalized over unphosphorylated ERK) are expressed as % of the respective control, in absence or presence of ET-1 and absence or presence of the solvent DMSO (which was required to dissolve BQ-788). Given are means + SEM of n=4 independent Western blots. Significance of differences: *P<0.05, **P<0.01, ***P<0.001 vs respective Ctr..

3.4.10. Role of the Rho-kinase in ET-1-induced activation of ERK1/-2 MAPK

In order to investigate whether Rho-kinase is involved in ET-1 induced activation of ERK1/-2 MAPK pathways, interaction experiments using the Rho kinase inhibitor, Y-27632 (3 µM) were performed. As shown in figure 3-35, under control conditions, Y-27632 (3 µM) by its own did not affect basal phospho-p42/44 levels; however the increase in phosphorylated ERK1/-2 MAPK induced by 10 nM ET-1 was partially inhibited by 3 µM Y-27632. In other words, in presence of Y-27632 Rho-kinase inhibitor, ET-1 still induced a significant increase in phosphorylated ERK1/-2 MAPK, but its effects was significantly attenuated.

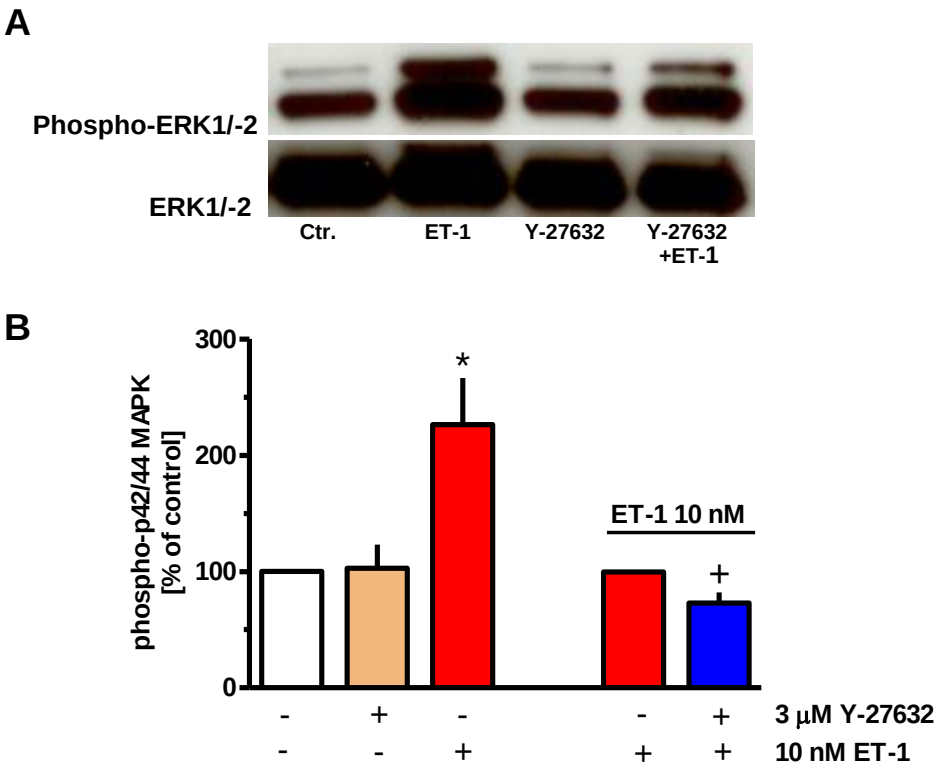


Figure 3-35: Western blot analysis of phospho-p42/44 (ERK1/-2) MAPK in MRC-5 human lung fibroblasts. Cells were cultured in 55 mm dishes to nearly confluence, serum-starved for 24 h and exposed to 10 nM ET-1 or vehicle (Ctr.) for 5 min (as indicated). The Rho kinase inhibitor (Y-27632, 3 µM) was present 30 min prior to exposure to ET-1. In controls, cells were treated with solvent at the respective time points (i.e. 5 min and additionally 30 min before cell lysis). Cell lysates were prepared and 20 µg were separated in 4-12% acrylamide Tris–glycine gels, immobilized to PVDF membranes and detected by antibodies specific for phosphorylated and unphosphorylated ERK. (A) Samples of original Western blots. (B) Densitometrical quantification of the p44/42 bands was performed and values (arbitrary units, normalized over unphosphorylated ERK) expressed as % of the respective control and in absence or presence of ET-1. Given are means + S.E.M of n=3 independent experiments. Significance of differences: *P<0.05 vs respective Ctr.; $^{+}$P<0.05 vs respective value in absence of Y-27632.

3.5. Characterization of the involvement of signal pathway of ET-1-mediated collagen synthesis stimulation in human lung fibroblasts

3.5.1. Effect of pertussis toxin on ET-1-mediated collagen synthesis stimulation

Since ET-1 receptors belong to the GPCR subfamily (Arai *et al.*, 1990; Sakurai *et al.*, 1990) and the preceding results clearly demonstrated that ET-1 mediates an increase in collagen synthesis via ET-A receptor subtype in human lung fibroblasts, it was investigated whether the stimulatory effect of ET-1 on collagen synthesis in human lung fibroblasts is mediated via $G_{\alpha i}$-coupled receptor like the effect of muscarinic receptors (Haag *et al.*, 2008a). Pertussis toxin (PTX), an exotoxin produced by Bordetella pertussis was used, as a pharmacological tool to elucidate the involvement of $G_{\alpha i}$ proteins. For this purpose, serum starved MRC-5 fibroblasts were pre-incubated for 3 hours with 50 ng/ml PTX, followed by measuring the effects of 100 nM ET-1 or 10 µM oxotremorine on collagen synthesis as determined by [^{3}H] proline incorporation. Under control conditions, ET-1 and oxotremorine increased the [^{3}H]-proline incorporation to 173% and 146%, respectively. PTX reduced basal [^{3}H]-proline incorporation by about 37% and completely inhibited the effect of 10 µM oxotremorine, but not that of ET-1. When expressed in relation to the respective untreated control values as shown in figure 3-36, the effect of ET-1 was largely attenuated, but when expressed in relation to the respective (somewhat reduced) PTX value, an increase to 169% is seen, i.e. effect similar to that observed with control condition.

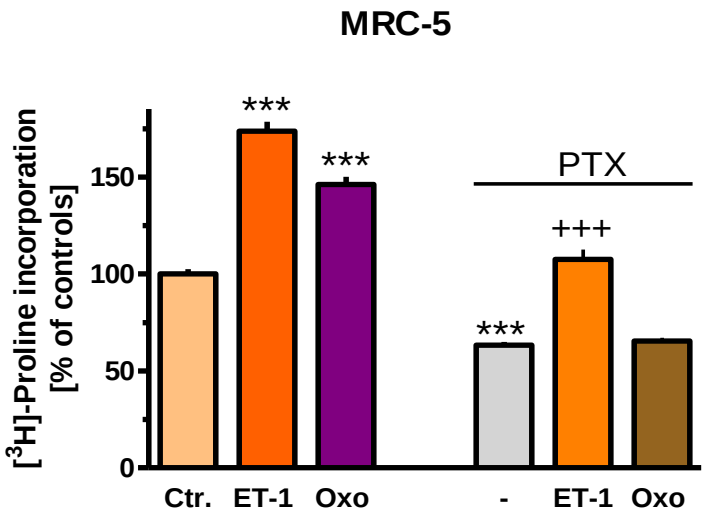


Figure 3-36: Effects of ET-1 (10 nM), oxotremorine (Oxo, 10 µM) and/or pertussis toxin (PTX, 50 ng/ml) on [^{3}H]-proline incorporation in MRC-5 human lung fibroblasts. 10^5 cells were seeded in 12-well dishes. Cells were first cultured for 24 h in presence of 10% FCS, followed by additional 24 hours under FCS-free conditions. Thereafter, [^{3}H]-proline (37 kBq) was added alone or in combination with test drugs, and cells were cultured for further 24 hours. PTX was present 3 hours prior to the exposure to ET-1 or Oxo. Radioactivity incorporated in cellular and extracellular protein was determined and expressed as % of the mean value of the Ctr. of each cell preparation. Given are means + S.E.M of n=9 independent experiments. Significance of differences: ***P<0.001 vs Ctr.; $^{+++}$P<0.001 vs respective value in presence PTX or ET-1 alone.

3.5.2. Effect of pertussis toxin on ET-1-induced activation of the ERK1/-2 MAPK pathway

In order to characterize the involvement of G_i proteins in ET-1 induced ERK1/-2 activation, the effect of pertussis toxin (PTX) on ET-1 induced phosphorylation of ERK1/-2 was studied. As shown in figure 3-38, PTX (50 ng/ml) alone caused a reduction in phospho-ERK1/-2 levels by about 37% and largely attenuated the increase in phospho- ERK1/-2 induced by 10 µM oxotremorine, but did not have clear effect on that of ET-1 (Fig. 3-38). When expressed in relation to untreated controls or in relation to the respective ET-1 values, the increase in phospho-ERK1/-2 induced by 100 nM ET-1 was somewhat reduced by PTX, but when expressed in relation to the respective PTX values, the effect of ET-1 on phospho-ERK1/-2 was similar to that observed in absence of PTX.

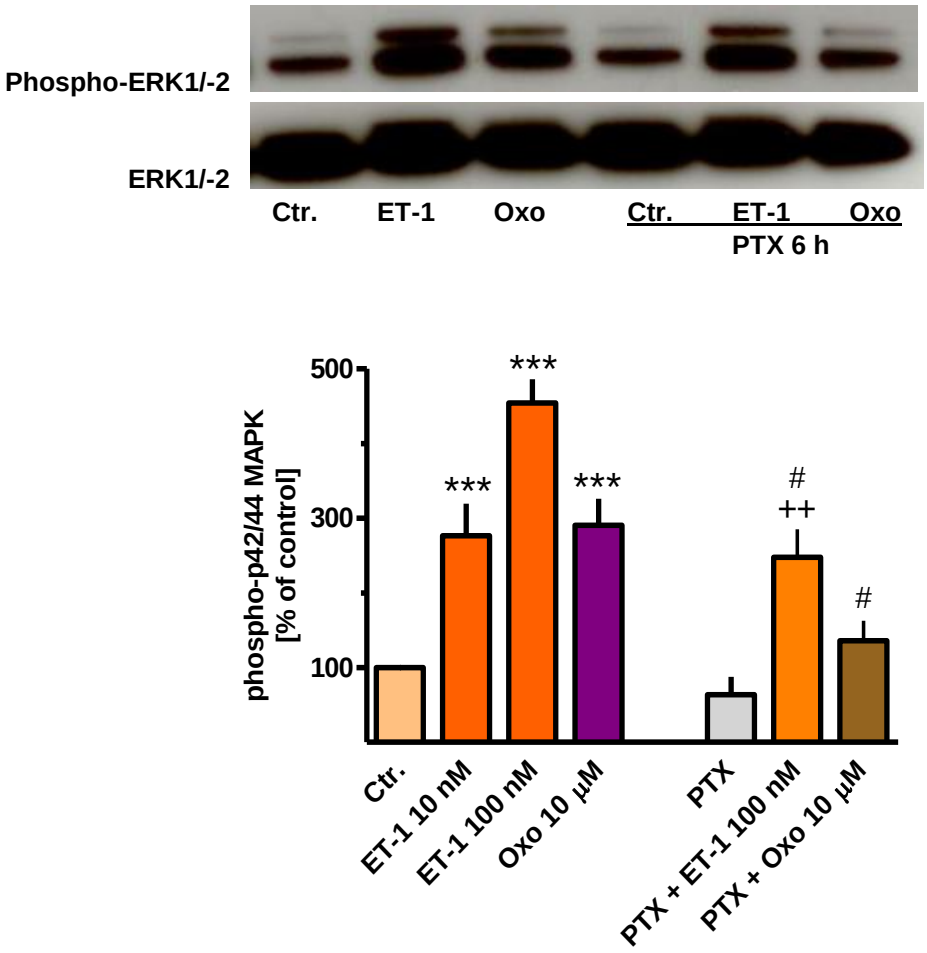


Figure 3-38: Western blot analysis of phospho-p42/44 (ERK1/-2) MAPK in MRC-5 human lung fibroblasts. Cells were cultured in 55 mm dishes to nearly confluence, serum-starved for 24 hours and exposed to 10 or 100 nM ET-1, 10 µM oxotremorine (Oxo), or vehicle (Ctr.) for 5 min (as indicated). In the respective experiments, pertussis toxin (PTX, 50 ng/ml) was present 6 hours prior to exposure to ET-1. In controls, cells were treated with solvent at the respective time points (i.e. 5 minute and additionally 6 hours before cell lysis). Cell lysates were prepared and 20 µg were separated in 4-12% acrylamide Tris–glycine gels, immobilized to PVDF membranes and detected by antibodies specific for phosphorylated and unphosphorylated ERK. (A) Samples of original Western blots. (B) Densitometrical quantification of the p44/42 bands was performed and values (arbitrary units, normalized over unphosphorylated ERK) are expressed as % of the respective control and in absence or presence of ET-1. Given are means + S.E.M of n=4–14 independent experiments. Significance of differences: ***P<0.001 vs respective Ctr.; $^{\#}$P<0.01 vs respective value in absence of PTX; $^{++}$P<0.01 vs respective value in presence of PTX alone.

3.5.3. Role of ERK1/-2 MAPK in ET-1-mediated stimulation of collagen synthesis

As previous results showed that ET-1 can activate ERK1/-2 in MRC-5 cells, it was tested whether ET-1 may stimulate collagen synthesis through the MAP kinase pathways. Therefore, interaction experiments with PD 098059, a specific inhibitor of the upstream kinase that activates ERK1/-2 MAP kinase were performed. As shown in figure 3-39, PD 098059 did not affect the basal [^{3}H]-proline incorporation, but the stimulatory effect of 10 nM ET-1 on [^{3}H]-proline incorporation was largely inhibited by 10 µM and prevented by 30 µM PD 098059.

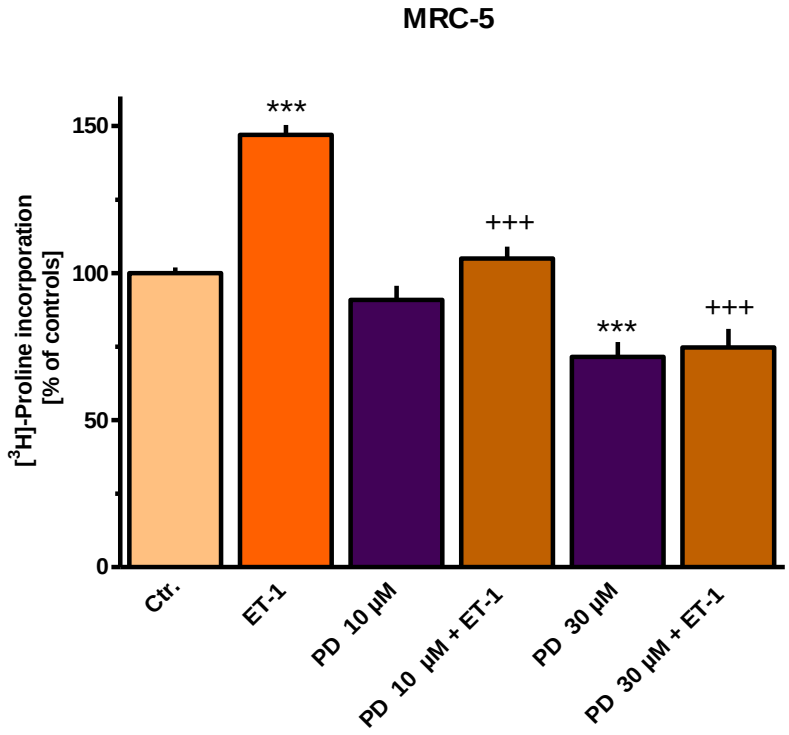
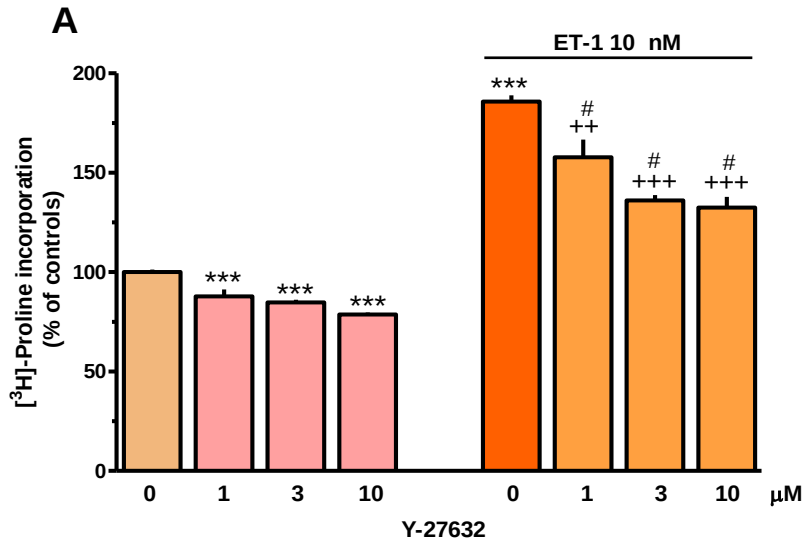


Figure 3-39: Effects of ET-1 (10 nM) and/or PD 098059 (PD, 10 and 30 µM) on [^{3}H]-proline incorporation in MRC-5 human lung fibroblasts. 10^5 cells were seeded in 12-well dishes. Cells were first cultured for 24 hours in presence of 10% FCS, followed by additional 24 hours under FCS-free conditions. Thereafter, [^{3}H]-proline (37 kBq) was added alone or in combination with test drugs, and cells was cultured for further 24 hours. In the respective experiments, PD was present 30 minute prior to the exposure to ET-1. 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 + S.E.M of n=9 independent experiments. Significance of differences: ***P<0.001 vs Ctr.; $^{+++}$P<0.001 vs. respective value in presence of ET-1 alone.

3.5.4. Role of Rho kinase in ET-1-mediated stimulation of collagen synthesis

In order to investigate whether Rho kinase is involved in endothelinergic stimulation of collagen synthesis in MRC-5 fibroblasts, effects of the Rho-kinase inhibitor Y-27632 on the stimulatory effect of ET-1 on collagen synthesis were studied. As shown in figure 3-40A, ET-1 (10 nM) enhanced the [^{3}H]-proline incorporation to 186% while the Rho-kinase inhibitor Y-27632 reduced the basal level of [^{3}H]-proline incorporation significantly to 87%, 84% and 78% at 1, 3 and 10 µM concentration, respectively. The stimulatory effect of ET-1 (10 nM) on collagen synthesis was largely attenuated by Y-27632 at 1, 3 and 10 µM concentration as shown by the reduction in the [^{3}H]-proline incorporation to 157%,136%, 132%, respectively in relation to untreated controls (Fig. 3-40A). When expressed in relation to the respective value the of 3 and 10 µM Y-27632, the increase caused by ET-1 was partially but significantly attenuated to 159 % and 168%, respectively, but not affected by 1 µM Y-27632 (Fig. 3-40B). In another word, in presence of both drugs, ET-1 still induced a significant increase in [^{3}H]-proline incorporation compared to 3 µM or 10 µM Y-27632 alone, i.e. ET-1 exerted still a significant stimulatory effect, although smaller than evoked by ET-1 alone.



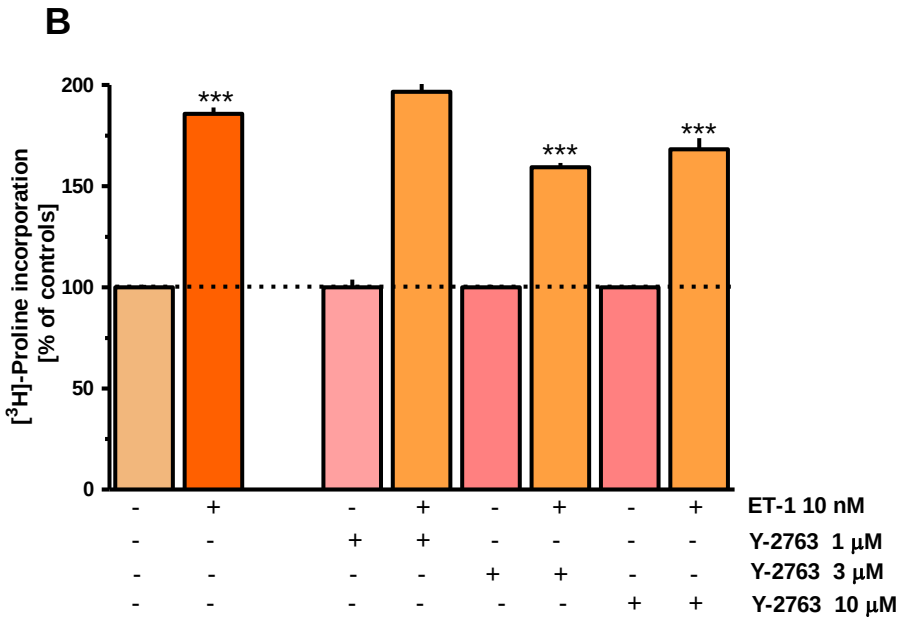


Figure 3-40: Effects of ET-1 (10 nM) and/or Rho kinase inhibitor (Y-27632, at the concentrations given) on [^{3}H]-proline incorporation in MRC-5 human lung fibroblasts. 10^5 cells were seeded in 12-well dishes. Cells were first cultured for 24 hours in presence of 10% FCS, followed by additional 24 hours under FCS-free conditions. Thereafter, [^{3}H]-proline (37 kBq) was added alone or in combination with test drugs, and cells was cultured for further 24 hours. In the respective experiments, Y-27632 was present 30 minute prior to the exposure to ET-1. 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 + S.E.M of n=9 independent experiments. Significance of differences: *P<0.05, ***P<0.001 vs respective control; ++P<0.01, +++P<0.001 vs. respective value in presence of ET-1 alone; $^{\#}$P<0.001 vs. respective value in presence of Y-27632 alone

4. Discussion

4.1. Expression of endothelines and their receptors in human lung fibroblasts

As outlined already in the introduction, endothelins (ET-1, ET-2 and ET-3) represent a family of small peptides of 21 amino acids. They are synthesised as biological inactive large precursor proteins (ppET), which are proteolytically cleaved to yield the bioactive form of 21 peptides. They are each encoded by a separate and unique gene (Inoue *et al.,* 1989; Yanagisawa *et al.,* 1989), are expressed in different cells and tissues with different proportions.

The present study demonstrated by using quantitative real time PCR that mRNA encoding for ppET-1 and ppET-2 was clearly expressed in MRC-5 human lung fibroblast cell line and phLFb, whereas no transcript was detected for ppET-3. Nevertheless in both MRC-5 fibroblasts and phLFb cells, there were different expression levels of mRNA for ppET-1 and ppET-2. The expression levels of ppET-1 mRNA exceeded 100-fold those of ppET-2 in both MRC-5 fibroblasts and phLFb (Fig. 3-1). This indicates that ET-1 is the predominant ET in human lung fibroblasts. Moreover, under similar culture condition, the transcript levels of ppET-1 and ppET-2 were approximately five times higher in MRC-5 fibroblasts than in phLFb.

Both subtypes of ET receptors, ET-A and ET-B, were found to be expressed in MRC-5 and primary cells. The expression levels of mRNA for ET-A and ET-B receptors were approximately equal in MRC-5 cell, whereas in primary cells, the levels of mRNA for ET-B receptor were substantially higher than those for ET-A. This is consistent with previous observations (Gallelli *et al.,* 2005).

In addition, the expression of ET receptors (ET-A and ET-B) was confirmed at the protein level by Western blot analysis, using antibodies directed against ET-B and ET-A receptors. A band of approximately 70 kDa, the expected size for ET-B receptors was detected, and an additional smaller protein band was also observed, that may reflect a cellular degradation product still recognized by the antibody. The immunoblot for ET-A showed also two bands, both smaller than the expected size. Nevertheless, the larger band appears to be an ET-A receptor signal as it corresponds to a specific band of about 45 kDa observed in ET-A receptor transfected cells (reference sheet of Santa Cruz).

4.2. Regulation of ET expression and release in human lung fibroblasts

4.2.1. Effect of TGF-β on ET expression

The regulation of ET-1 expression takes place principally at the mRNA level that involves both transcriptional and posttranscriptional mechanisms (Inoue *et al.*, 1989; Rodriguez-Pascual *et al.*, 2003; Mawji *et al.*, 2004). Expression of ET-1 mRNA is constitutive, but potentially regulated by a variety of biological and pharmacological factors (Miyauchi *et al.*, 1999). ET-1 gene expression can be affected by a number of factors including TGF-β, which is one of the most potent regulators of ET-1 levels. TGF-β is a pro-fibrotic cytokine, which is thought to be one of the key mediators in tissue remodeling associated with chronic inflammatory and obstructive airway diseases (Königshoff *et al.*, 2009; Datta *et al.*, 2011; Halwani *et al.*, 2011). TGF-β has been reported to cause an increase in ET-1 mRNA and peptide levels in different type of cells, including primary human fetal lung fibroblasts (IMR-90) (Kulasekaran *et al.,* 2009), human lung fibroblasts (Shi-Wen *et al.,* 2006) and aortic endothelial cells (Markewitz *et al.,* 2001).

The current study confirmed that TGF-β is a strong stimulator of ET-1 expression; it caused an almost nine-fold increase of ppET-1 transcription in MRC-5 cells and a more than fifteen-fold increase in phLFb. The stimulatory effect of TGF-β was rapid in onset and did not show any fatigue during prolonged exposure (the longest period studied being one week). This finding suggests that elevation of TGF-β in pathological conditions might be accompanied by a sustained up-regulation of ET-1 expression. For example, both TGF-β and ET-1 have been reported in several studies to be highly expressed in the lungs of patients with pulmonary fibrosis (Cambrey *et al.,* 1994; Khalil *et al.,* 1996; Abraham *et al.,* 1997; Sime *et al.,* 1997; Mutsaers *et al.,* 1998; Wendel *et al.,* 2004). Taken together, the observations in the present study suggest that up-regulation of ET-1 in human lung fibroblasts by TGF-β may play an important role in fibrotic airway remodelling processes.

Furthermore, TGF-β has been shown in this study to have different effects on the expression of ET isoforms in both, MRC-5 cells and phLFb. The stimulatory effect of TGF-β on the expression of ET is confined to ET-1, as TGF-β caused even a reduction of the already low levels of ppET-2 mRNA (Fig. 3-3B) and did not induce the expression of ppET-3 mRNA.

4.2.2. Effect of TGF-β on ppET-1 mRNA stability and/or transcription

In general, ET-1 mRNA appears to be rather labile, having an intracellular half life of less than an hour (Inoue *et al.,* 1989). In the present study, the half-life of ppET-1 mRNA was about 30 minutes (Fig. 3-4A), indicating a relatively high turnover rate resulting in a close relation between mRNA levels and ppET-1 gene transcription. Such a rapid turnover of the ET-1 transcript permits stringent control over its expression in response to extracellular stimuli. Therefore, ppET-1 mRNA levels are expected to reflect immediately changes in ppET-1 gene transcription. In line with this conclusion, there was a marked increase (about sevenfold) in ppET-1 mRNA levels already after one hour of exposure to TGF-β (Fig. 3-4B). An essential characteristic of ppET-1 mRNA is the existence of a number of AUUUA motifis in the 3′ untranslated region. The AU motifis are known to mediate selective mRNA degradation through accelerated deadenylation, which could be contribute to the lability of ET-1 mRNA (Mawji *et al.,* 2004).

Furthermore, the up-regulation of ppET-1 mRNA expression induced by TGF-β was prevented by the transcriptional inhibitor actinomycin D, and this was not a result of actinomycin D toxicity, since GAPDH steady-state mRNA levels (reference gene) were not affected by this substance. This indicates that an increase in transcription of the ET-1 gene might be responsible for the enhanced mRNA levels rather than an enhanced stability of the transcript.

In an attempt to investigate whether the stimulatory effect of TGF-β on ET-1 mRNA involved *de-novo* cellular protein synthesis, experiments using the protein synthesis inhibitor cycloheximide were performed. The observation that cycloheximide alone cause a rapid increase in ppET-1 mRNA indicates, that basal ppET-1 gene expression in human lung fibroblasts is under inhibitory control of short-living regulatory peptides, not yet identified, which appear also to counteract the stimulatory effect of TGF-β, as the TGF-β response was markedly potentiated in the presence of cycloheximide (Fig. 3-5B). Interestingly, the rapid onset of the stimulatory effect of TGF-β on ppET-1 mRNA expression occurred also in presence of cycloheximide, indicating that the induction of ppET-1 mRNA does not required *de novo* protein synthesis. This supports the conclusion that the up-regulation of ppET-1 mRNA expression by TGF-β is the result of a direct regulation of ppET-1 mRNA

gene transcription by TGF-β signalling pathways and does not involve the up-regulation of other regulatory proteins.

4.2.3. Effect of TGF-β on ET-1 release

MRC-5 fibroblasts appear to release ET-1 constitutively, as accumulation of the biologically active ET-1 in culture medium was observed under an un-stimulated condition. However, there was a strong increase in ET-1 accumulation in TGF-β stimulated cells, indicating that the TGF-β-mediated up-regulation of ET-1 mRNA expression was accompanied by an increase in synthesis and release of ET-1. Strikingly, the maximum level of ET-1 accumulation caused by TGF-β was observed at TGF-β concentrations of 0.1 - 0.3 ng/ml which induced only sub-maximal increase in ppET-1 mRNA. The further strong increase in ppET-1 mRNA caused by higher concentrations of TGF-β did not result in a higher rate of accumulation (compare figures 3-3A and 3-7A). A simple relationship between the levels of ppET-1 mRNA and ET-1 accumulation may not be expected, since ET-1 accumulation is determined by a number of processes. These include translation into the precursor protein, its intracellular processing to big-ET-1, release of big-ET-1 and extracellular conversion to ET-1 and finally degradation of the active peptide.

Furthermore, the conversion of exogenous big ET-1 into the active peptide occurred in a concentration- and time-dependent manner, maximally at a concentration of 100 nM over 6 hours exposure time (Fig. 3-8). Since the concentrations of ET-1, which accumulated in presence of exogenous big-ET-1, were about 40-times higher than those seen in media from cells stimulated with TGF-β (Fig. 3-7A), it can be excluded that a limited ET converting enzyme activity may account for the above discussed dissociation between ppET-1 mRNA and accumulation of ET-1. Thus, limited translation and/or limited processing of the precursor protein may account for the non-linear relationship between mRNA levels and accumulation of the active peptide.

4.2.4. Role of ET receptor subtypes in TGF-β-induced expression and release of ET-1

A potential role of ET receptors (ET-A and ET-B) on TGF-β-induced expression and release of ET-1 in MRC-5 cells was studied by using the non selective ET receptor antagonist bosentan as well as selective ET-A and ET-B receptor antagonists, which have been demonstrated to be important tools to examine the role of each receptor type (Davenport, 2002). The concentrations of the antagonists were chosen to be selective for the respective receptor. As the affinity of BQ-788 at the ET-B receptor is about ten-fold higher than that of BQ-123 at the ET-A receptor (Ishikawa *et al*., 1994), at the concentration used (0.1 µM BQ-788 and 1 µM BQ-123) a similar blockade of the respective ET receptor subtype should have been achieved by each antagonist.

The up-regulation of ppET-1 mRNA expression and the accumulation of ET-1 induced by a sub-maximal concentration of TGF-β (0.03 ng/ml) were significantly attenuated by the non selective ET receptor antagonist bosentan, indicating that ET-1 may contribute in a kind of auto-regulatory positive feedback, to the regulation of its own expression and release. It can be conclude that ET-A receptors mediated these effects on the expression of ppET-1 in human lung fibroblasts, since the selective ET-A antagonist BQ-123, but not the ET-B selective antagonist BQ-788 mimicked the effect of bosentan. This indicates that TGF-β-induced up-regulation of ppET-1 expression is augmented by concomitant ET-A receptor activation. Thus, an auto-regulatory positive feedback mediated via ET-A receptors appear to be involved in the regulation of ET-1 expression and release. A similar ET-A receptor-mediated feedback mechanism was also described for the TNF-α-induced up-regulation of ET-1 in human airway smooth muscle cells (Knobloch *et al.,* 2009).

It was observed that neither antagonist significantly changed basal levels of ppET-1 mRNA and release in MRC-5 fibroblasts, indicating that the autocrine and/or paracrine activation of ET-A and ET-B receptors does not regulate basal ppET-1 transcription and release in human lung fibroblasts.

4.2.5. Role of canonical (Smad) and non-canonical (MAPK, Rho G-protein) signaling in TGF-β-induced ppET-1 mRNA expression

Activation of canonical (Smad) and non-canonical (MAPK, Rho G-protein) signalling pathways involved in TGF-β-mediated gene transcription have been reported in various studies (Rodriguez-Pascual *et al.,* 2003; Leivonen *et al.,* 2005; Castanares *et al.,* 2006; Star *et al.,* 2009; Shimada *et al.,* 2011). As outlined in the introduction, eight Smad proteins family members have been identified, which play a critical role in the transduction of receptor signals to particular target genes in the nucleus (Attisano *et al.,* 2001). They are considered to be important mediators of TGF-β signalling (Heldin *et al.*, 1997; Attisano *et al.*, 2000; Massague *et al.*, 2000a). Smad3 is part of a canonical signalling pathway of TGF-β, which is considered to be a mediator of TGF-β-induced regulation of gene transcription (Attisano *et al.,* 2002). In endothelial cells, it was shown that, the TGF-β-Smad pathway was involved in the regulation of ET-1 mRNA expression (Rodriguez-Pascual *et al.,* 2003; Castanares *et al.,* 2006).

In the present study the TGF-β-Smad3 pathway appears also to be crucially involved in TGF-β-induced up-regulation of ppET-1 mRNA in MRC-5 fibroblasts, as indicated by the strong inhibitory effect of SIS3, a specific inhibitor of Smad3. The observation that SIS3 markedly reduced basal level of ppET-1 mRNA expression indicates that, the TGF-β-Smad3 signalling pathway was activated already under control culture condition. In contrast to these data, mutation of the Smad element in the ET-1 promoter did not affect TGF-β-mediated induction of ET-1 in lung fibroblasts (Shi-Wen *et al.,* 2006).

In addition to Smad3, the ERK MAPK signaling pathway has been shown to play also a role in TGF-β signalling (Zavadil *et al.,* 2001; Leask *et al.,* 2003; Leivonen *et al.,* 2005) and to contribute to TGF-β-induced gene expression (Robinson *et al.*, 1997). In the present study, inhibition of the ERK MAPK pathway by the MEK inhibitor PD 098059 (Alessi *et al.,* 1995; Dudley *et al.,* 1995) had no effect on the basal level of ppET-1 mRNA expression after 4 or 8 hours of exposure, indicating that ERK1/-2 MAPK was not involved in the short term regulation of basal ppET-1 mRNA expression in MRC-5 fibroblasts. Moreover, TGF-β-induced increases in ppET-1 mRNA expression was not affected by the MAPK inhibitor, indicating that ERK1/-2 MAPK is not required for TGF-β-induced ppET-1 mRNA expression.

Interestingly however, prolonged inhibition of the MAPK pathway by 24 hours exposure to PD 098059 caused a marked increase in ppET-1 mRNA and had additive effects with TGF-β. The mechanism by which inhibition of MAPK pathway caused up-regulation of ppET-1 mRNA remains unknown. Whether an altered gene expression or effects on the stability of the mRNA are involved has to be investigated in further studies.

As outlined in the introduction, Rho kinase is a member of the Ras superfamily of small GTP-binding proteins and recognised as one of the downstream effectors of the small Rho GTPase Rho A. RhoA-Rho kinase activation controls a variety of cellular functions including, the transcriptional regulation of gene expression (Etienne-Manneville *et al.*, 2002; Shimokawa *et al.*, 2007). Participation of Rho kinase-dependent signalling in TGF-β-induced ppET-1 mRNA expression has been demonstrated in hepatic stellate cells (Shimada *et al.,* 2011).

The current observation that the Rho-kinase inhibitor Y-27632 (Ishizaki *et al.,* 2000; Shimokawa *et al.,* 2007) concentration dependently reduced the basal ppET-1 mRNA levels indicates that, Rho is involved in the basal regulation of ppET-1 mRNA expression in human lung fibroblasts. However, it appears not to be involved in the regulation of ppET-1 transcription by TGF-β, as Y-27632 did not affect the TGF-β-induced increase in ppET-1 mRNA expression.

4.2.6. Role of β_2-adrenoceptors and muscarinic receptors in the ET-1 expression

Another aim of the present study was to illuminate a possible role of β_2-adrenoceptors and muscarinic receptors in the regulation of ET-1 expression.

4.2.6.1. Effect of β_2-adrenoceptor agonist on ET-1 expression

β–Adrenoceptors are involved in the regulation of crucial cellular functions of human lung fibroblasts which express exclusively the β_2-subtype (Lamyel *et al*., 2011).

In the present study, it was investigated whether the expression of ppET-1 mRNA in human lung fibroblasts is regulated by β-adrenergic mechanisms. The

highly selective, long acting β_2-adrenoceptor agonist olodaterol (Bouyssou *et al.,* 2010) caused a marked reduction of the expression of ppET-1 mRNA in MRC-5 fibroblasts, indicating that ET-1 expression is regulated by inhibitory β_2-adrenoceptors in these cells. Furthermore, in line with the expression pattern of β-adrenoceptors in human lung fibroblasts, the inhibitory effects of olodaterol on ppET-1 mRNA expression was antagonised by the β_2-adrenoceptor selective antagonist ICI 118,551, whereas the β_1-adrenoceptor antagonist CGP 20712 was ineffective.

As outlined in the introduction, β-adrenoreceptors typically couple to G_s and cause increases in cellular cAMP concentration through activation of adenylyl cyclase. Intracellular cAMP regulates various cellular functions primarily through two downstream effectors, PKA, the classic effector of cAMP (Skalhegg et al., 2000), and the alternative cAMP effectors Epac of which two variants, Epac-1 and -2, have been identified (de Rooij et al., 1998; Kawasaki et al., 1998). These effectors (Epac and PKA) have been shown to act independently on distinct cellular functions in human lung fibroblasts, such as proliferation and collagen synthesis (Huang *et al.*, 2008; Haag *et al.*, 2008b). In the present study, the selective PKA agonist 6-Bnz-cAMP effectively inhibited the expression of ppET-1 mRNA, suggesting that the effect of β_2-adrenoceptors may be mediated via PKA. Interestingly, the Epac agonist 8-CPT-2′-O-Me-cAMP (Bos, 2006; Holz *et al.*, 2008; Poppe *et al.*, 2008) also significantly reduced the expression of ppET-1 mRNA, but this reduction was substantially smaller than that caused by the PKA agonist 6-Bnz-cAMP. In previous studies in our laboratory using the same cells and the same concentrations of 6-Bnz-cAMP and 8-CPT-2'-O-Me-cAMP, these agonists mediated a strong and selective inhibition of either the synthesis of collagen or cell proliferation, respectively (Haag *et al.,* 2008b). Collective theses observations indicate that the cellular transmission of β_2-adrenoceptor–cAMP-mediated inhibition of the transcriptional regulation of ET-1 expression could be by the engagement of both PKA and Epac, but PKA appears to play the major part.

Since TGF-β was shown to up-regulate ET-1 gene expression, and the selective β_2-adrenoceptor agonist (olodaterol) down-regulated the expression of ET-1, the opposing role of TGF-β and β_2-adrenoceptors was further studied in interaction experiments. Activation of β_2-adrenoceptor by olodaterol was able to oppose the TGF-β-induced up-regulation of ET-1, but only when a sub-maximally effective

concentration of TGF-β (0.3 ng/ml) was applied. The marked up-regulation of ET-1 by TGF-β in the maximally effective concentration of 1 ng/ml was no longer affected by olodaterol. Interestingly, interaction experiments with the PKA and Epac agonists and TGF-β showed similar results. The PKA and Epac agonists were able to oppose the effect of a sub-maximally effective concentration of TGF-β (0.3 ng/ml), but not that of a maximally effective concentration of TGF-β (1 ng/ml). Noticeably, the effect of the PKA agonist tends to be stronger than that of the Epac agonist. Since TGF-β caused a large suppression of the expression of β_2-adrenoceptors in human lung fibroblasts (Ahmedat *et al.*, 2012), the loss of inhibitory effects of β-adrenoceptor on ppET-1 expression caused by TGF-β appear to result of both, down-regulation of receptors and uncoupling of the β-adrenoceptor-downstream signalling cascade.

4.2.6.2. Effect of muscarinic cholinergic receptor agonist on ET-1 expression

Muscarinic receptors and β_2-adrenoceptors generally mediate opposing regulatory effects and this seems to apply also for human lung fibroblasts where muscarinic receptors and β_2-adrenoceptors mediate opposing effects on various pro-fibrotic features. For example, in these cells, proliferation and collagen synthesis are stimulated by muscarinic receptors activation (Matthiesen *et al.,* 2006; Haag *et al.,* 2008a), but inhibited by activation of β_2-adrenoceptors (Lamyel *et al.,* 2011).
In accordance with this, the present study shows that the muscarinic receptor agonist oxotremorine-mediated a marked stimulatory effect on the expression of ppET-1 mRNA in MRC-5 fibroblasts. Moreover, the stimulatory effect of oxotremorine was opposed by concomitant activation of β_2-adrenoceptors with olodaterol. Altogether, these observations suggest that up-regulation of ET-1 may contribute to the pro-fibrotic effects of muscarinic stimuli. Thus, reduction of cholinergic driven induction of ET-1 expression could contribute to long-term beneficial effects of long-acting muscarinic antagonists such as tiotropium.

Additionally, the present study demonstrated that the effects of the muscarinic agonist oxotremorine and those of TGF-β seemed to parallel in MRC-5 fibroblasts, as both mediated a strong stimulatory effects on ppET-1 mRNA expression. The muscarinic receptor agonist showed however only limited additive effects to that of TGF-β, an additivity on ppET-1 transcription was observed only with the relatively

low concentrations of 0.1 ng/ml TGF-β. However, in presence of 0.3 ng/ml TGF-β, still not a maximally effective concentration, oxotremorine no longer caused a significant further increase in ppET-1 mRNA expression.

Acetylcholine, a classic neurotransmitter has been reported to be synthesized also by many non-neuronal cells including fibroblasts (Wessler *et al.,* 1998; Wessler *et al.,* 2008). However, the lack of effect of tiotropium on ET-1 expression, both under basal culture conditions as well as in presence of TGF-β, excludes a role of endogenous acetylcholine in the control of ET-1 expression. The observation that bosentan attenuated the muscarinic receptor agonist-induced stimulation of ET-1 expression indicates, that similar to the above discussed observations on the mode of actions of TGF-β, a paracrine/autocrine positive feedback mechanism mediated via ET receptors is involved in oxotremorine-mediated up-regulation of ppET-1.

4.3. Effects of TGF-β on the expression of ET receptor subtypes in human lung fibroblast

In both phLFb and MRC-5 fibroblasts ET-B receptor transcription appears to be robustly down-regulated by TGF-β, whereas ET-A receptor appears to be highly constitutive. These data show that TGF-β modulates the expression of ET receptor subtypes differently in human lung fibroblasts. Since, TGF-β did not affect ET-A expression, it can be concluded that ppET-1 and ET-A receptor mRNA are not under the same regulation. Previously, it had also been observed in other tissues and cells such as rat mesenteric artery and alveolar epithelial cells that the ET-A receptor is constitutively expressed, whereas the ET-B receptor expression is strongly regulated (Uddman *et al.,* 2002; Jain *et al.,* 2007).

4.4. Functional role of ET-1 and its receptors in human lung fibroblasts

Another aim of the present study was to illuminate the role of the ET-1 and its receptor subtypes in human lung fibroblasts in specific cellular functions such as proliferation, collagen synthesis or modulation of phenotype. These cellular functions are of pathophysiological relevance in the development of lung diseases such as asthma, chronic obstructive disease and idiopathic pulmonary fibrosis. In fact, these

pathological conditions have a common tendency toward enhanced cellular activities including fibroblast proliferation, differentiation into myofibroblast and collagen synthesis leading to an increase accumulation of ECM. For example, lung fibrosis is characterized by abnormal proliferation, activation of lung fibroblasts and production of extracellular matrix that results in the excessive deposition of extracellular matrix proteins (Crouch, 1990; Thannickal *et al.*, 2004; Datta *et al.*, 2011).

4.4.1. Effects of ET-1 and ET receptor subtype antagonists on proliferation

The present study demonstrated that ET-1 exerted proliferative effects in both MRC-5 fibroblasts and phLFb.

In MRC-5 fibroblasts, under the cultured conditions depicted in figure 3-20B (condition A), ET-1 caused only a slightly stimulatory effect on the proliferation as measured by [^{3}H]-thymidine incorporation but, on the other hand, bosentan caused a significant reduction in the basal [^{3}H]-thymidine incorporation, suggesting that under these culture conditions, endogenously released ET-1 exerted a clear proliferative effect. This is in line with the observed of constitutive synthesis of ET-1 in these cells. A mitogenic effect of endogenous ET-1 has also been demonstrated on human pulmonary artery smooth muscle cells and rat cardiomyocytes (Ito *et al.,* 1993; Wort *et al.,* 2001). Although ET-1 alone had only a small stimulatory effect, ET-1 was able to surmount the inhibitory effect bosentan, indicating a competitive interaction between ET-1 and bosentan, and supporting that the inhibitory effect of bosentan was indeed mediated by specific blockade of ET receptors. Since only BQ-123, but not BQ-788 mimicked the effect of bosentan, it is concluded that ET-A receptors mediate the proliferative stimulus.

In phLFb, the stimulatory effect of ET-1 on proliferation was stronger than in MRC-5 cells, but the inhibitory effect of bosentan and BQ-123 on the proliferation was smaller, in addition to this BQ-788 was again ineffective. These functional observations suggesting a lower endothelinergic tone in phLFb compared with MRC-5 cells are in accordance with lower expression levels of ET-1 in phLFb under standard cultured conditions. Like in MRC-5 cells, the inhibitory actions of bosentan and BQ-123 were also surmountable by exogenous ET-1 in phLFb.

Additionally, in both phLFb and MRC-5 cells, the proliferative effect of exogenous ET-1 was somewhat more pronounced when the cells were starved after five hours after the cell dissemination (condition B) (Fig. 3-20C), i.e. conditions, where a lower endogenous ET-1 tone might have been present. Moreover, in both cell types (phLFb and MRC-5 cells), exogenous ET-1 exerted a stronger proliferative effect when ET-1 was applied immediately after cells dissemination. Noteworthy, under these conditions the maximum proliferative effect of ET-1 was achieved at lower concentrations of ET-1 (Fig. 3-20D), again in line with the conclusion that under these conditions the endogenous ET-1 tone may be lower than under the standard culture conditions (condition A).

Since DNA synthesis may occur without cell division, cell numbers were estimated in order to ascertain that the increase in DNA synthesis induced by ET-1 in human lung fibroblast cells reflects an increase in cell proliferation. In agreement with the observations with the [^{3}H]-thymidine incorporation method, it was found that ET-1 caused an increase in the number of cells, an effect inhibited by bosentan.

The present observation with regard to the lack of ET-B receptors effects on human lung fibroblasts proliferation are in line with observations reported by Gallelli *et al.*, (2005) showing that in phLFb, proliferative effects of ET-1 mediated only through ET-A receptors, but contrast with the observations of Préfontaine *et al*., (2008) which reported that ET-A and ET-B receptor were involved in ET-1-induced proliferation in rat lung fibroblasts. The discrepancy may reflect species differences.
Collectively, these observations indicate that ET-1 exerts stimulatory effects on both DNA synthesis and proliferation of human lung fibroblasts via activation of ET receptors. Thus, ET-1 may contribute to airway remodelling, at least in part by stimulating bronchial fibroblasts to synthesize DNA and proliferate (Dubé *et al.*, 2000).

4.4.2. Role of Rho kinase in ET-1-induced proliferation

As outlined in the introduction, Rho kinase is the main effector of Rho. Rho, a member of the family of small G-proteins, has been shown to regulate a variety of cellular functions including cell proliferation in part via its target, Rho kinase

(Coleman *et al.*, 2004). Abnormal activation of the Rho/Rho-kinase pathway has been reported to play a role in various diseases including pulmonary fibrosis (Jiang *et al.,* 2012) and bronchial asthma (Wettschureck *et al.,* 2002). In a previous study, it has also been shown, that inhibition of Rho kinase abrogates ET-1-induced proliferation of rat tracheal smooth muscle cells (Yahiaoui *et al.,* 2006). In the present study, the Rho kinase inhibitor Y-27632 by itself inhibited the basal proliferation, but failed to inhibit ET-1-induced [^{3}H]-thymidine incorporation in MRC-5 fibroblast. These results indicate that, Rho is not involved in the ET-1-induced proliferation of MRC-5 fibroblast, although it mediates proliferative signals in these cells.

4.4.3. Role of ET-1 and its receptors in the regulation of collagen synthesis

After establishing that ET-1 stimulates the proliferation of human lung fibroblasts, it was interesting to further investigate whether ET-1 might also be involved in the regulation of collagen synthesis. Collagen is a major extracellular matrix component of the lungs and is vital for maintaining the normal lung architecture. The major component of collagen is proline and therefore the amount of radioactive [^{3}H]-proline incorporated into newly synthesised proteins provides a reliable index of total collagen synthesis. [^{3}H]-Proline incorporation method has often been used as a measure of collagen synthesis (Peterkofsky *et al.*, 1971; Guo *et al.*, 2004; Hafizi *et al.*, 2004; Rao *et al.*, 2008; Haag *et al.*, 2008a). Furthermore, in previous experiments carried out in our laboratory, it was confirmed by collagenase digestion that total radioactivity incorporated into proteins largely reflects *de novo* synthesis of collagen (Haag *et al.*, 2008a).

Previous studies have shown that ET-1 can stimulates collagen synthesis in a variety of cell types including human cardiac fibroblasts, human primary dermal fibroblasts, and human lamina cribrosa cells (Hafizi *et al.,* 2004; Horstmeyer *et al.,* 2005; Rao *et al.,* 2008). In the present study, it was found that ET-1 increased collagen synthesis in a concentration-dependent manner in both phLFb and MRC-5 fibroblasts. In accordance with the already discussed lower endogenous endothelinergic tone in phLFb compared to MRC-5 fibroblasts, none of the ET antagonists had any significant effect on basal [^{3}H]-proline incorporation in phLFb,

whereas in MRC-5 fibroblasts, bosentan and the ET-A antagonist BQ-123 caused a small, but significant reduction in [^{3}H]-proline incorporation. In both cell types, the inhibitory effect of bosentan and BQ-123 were surmountable by exogenous ET-1. On the other hand, BQ-788 did not affect the ET-1 response. Therefore, it can be conclude that the effects of ET-1 on collagen synthesis in human lung fibroblasts cells are mediated via the ET-A receptor subtype.

Furthermore, the stimulatory effect of ET-1 on collagen synthesis was also demonstrated by direct determination of collagen as measured by the QuickZyme collagen assay. Also using this method it could be observed that ET-1 caused in concentration-dependent manner a strong increase in collagen synthesis and the maximal effect of ET-1 observed on collagen synthesis as measured by the two different assay system ([^{3}H]-proline incorporation assay and QuickZyme collagen assay) was achieved at a concentration of 10 nM, i.e. the results obtained with the two different assay system were highly consistent.

4.4.4. Effect of ET-1 on COL I-α1 mRNA expression

In order to investigate whether the stimulatory effect of ET-1 on collagen synthesis involves an increase of collagen expression, the effect of ET-1 on the expression of COL I-α1 mRNA, a major collagen in lung fibroblasts (Kirk *et al.,* 1984) was studied. ET-1 caused an increase of COL I-α1 mRNA expression in MRC-5 fibroblasts and this effect was blocked by bosentan, indicating that ET-1 regulates COL I-α1 mRNA via activation of ET receptors. This observation supports the conclusion that an enhanced collagen gene transcription contributes to the ET-1-induced up-regulation of collagen synthesis in human lung fibroblasts.

4.4.5. Effects of ET receptor subtype antagonists on TGF-β-mediated collagen synthesis stimulation

TGF-β is one of the key cytokines in the regulation of extracellular matrix production (Bartram *et al.,* 2004). It has been demonstrated that TGF-β stimulates collagen synthesis in a variety of cells types, including human lung fibroblasts, human cardiac fibroblasts, and human dermal fibroblasts (Fine *et al.,* 1987; Hafizi *et al.,* 2004; Horstmeyer *et al.,* 2005).

In the present study, TGF-β proved to be a potent stimulator of collagen synthesis in human lung fibroblasts. Interestingly, bosentan and BQ-123, but not BQ-788 reduced the stimulatory effect of TGF-β on the collagen synthesis, indicating that endogenous ET-1 acting via ET-A receptors might be involved in TGF-β-induced stimulation of collagen synthesis. The present findings demonstrate that the observed up-regulation of ET-1 gene by TGF-β is of functional significance and may contribute to the pro-fibrotic effects of TGF-β. Moreover, the present observation further indicates, that ET-1 may be a downstream pro-fibrotic mediator of TGF-β in human lung fibroblasts. Consequently, ET-A receptor antagonists might be considered as potential drugs to treat fibrotic lung diseases.

4.4.6. Effect of ET receptor antagonist on muscarinic agonist-mediated collagen synthesis stimulation

Another aim of the present study was to identify a possible role of ET-1 in muscarinic receptor-mediated stimulation of collagen synthesis in human lung fibroblasts.

In line with a previous study which showed a muscarinic receptor-mediated stimulatory effect on collagen synthesis in human lung fibroblasts (Haag *et al.,* 2008a), the present data demonstrated that the muscarinic receptor agonist oxotremorine markedly up-regulated collagen synthesis in MRC-5 fibroblasts. Bosentan attenuated the oxotremorine-mediated stimulation of collagen synthesis, indicating that ET-1 may contribute to the pro-fibrotic effect of cholinergic stimuli, and that the observed up-regulation of ET-1 gene by oxotremorine is of functional significance.

Furthermore, the stimulatory effect of oxotremorine on collagen synthesis showed limited additivity to that of TGF-β. An additive effect of the muscarinic agonist was observed only with the low concentrations of 0.1 ng/ml TGF-β, but oxotremorine no longer exhibited a significant further effect in the presence of 0.3 ng/ml TGF-β, still a sub-maximally effective concentration. The additive effects of TGF-β and oxotremorine on collagen synthesis were consistent with the above discussed observations on ET-1 expression.

4.4.7. Role of ET-1 in myofibroblasts differentiation

In addition to proliferation, the transformation of human lung fibroblasts to myofibroblasts is a critical pathophysiological relevant feature which appears to play a key role in many lung disease including lung fibrosis (Kuhn *et al.*, 1991; Phan, 2002; White *et al.*, 2003). Myofibroblasts are fibroblast-like cell with smooth muscle cell characteristics, showing particular expression of α-SMA.

In agreement with previous studies (Singh *et al.,* 2008; Honda *et al.,* 2010; Lamyel *et al.,* 2011), MRC-5 fibroblasts cultured under standard conditions, showed significant expression levels of α-SMA, most likely induced by serum constituents of the culture medium. Unlike TGF-β, a well characterised stimulant of myofibroblast differentiation (Sandbo *et al.,* 2009; Honda *et al.,* 2010; Lamyel *et al.,* 2011; Milara *et al.,* 2012), exogenous addition of ET-1 caused only a minor, although significant increase in α-SMA. Bosentan caused a reduction in the expression of α-SMA protein by about 50% after 24 hours of exposure, suggesting that endogenously released ET-1 contributes to myofibroblast differentiation. However, ET-1, although up-regulated by exposure to TGF-β, seems not to be involved in TGF-β-induced differentiation of MRC-5 fibroblasts to the myofibroblast phenotype, as bosentan did not affect TGF-β-induced up-regulation of α-SMA protein expression (Fig. 3-25). These data suggest that, the endothelinergic system may be involved in the transformation of lung fibroblasts to myofibroblasts and that it may contribute to the increased pool of myofibroblasts that has been observed in lungs of patients with asthma and COPD. Since myofibroblasts are key effectors in airway remodelling processes associated with chronic obstructive and inflammatory airway diseases (Davies *et al.*, 2003; Westergren-Thorsson *et al.*, 2010), the inhibitory effect of bosentan on myofibroblast differentiation could be of particular therapeutic interest.

4.5. Characterization of the signal pathway involved in ET-1-mediated collagen synthesis stimulation in human lung fibroblasts

4.5.1. Effects of ET-1 and ET receptor subtype antagonists on ERK1/-2 MAPK activation

Previous studies carried out in our laboratory showed that in human lung fibroblasts activation of ERK MAPK pathway was crucially involved in the muscarinic receptor-mediated stimulation of proliferation and collagen synthesis (Matthiesen *et al.*, 2006; Haag *et al.*, 2008a). In the present study, similar to the muscarinic receptor agonist oxotremorine, ET-1 induced a significant and rapid, but transient, phosphorylation of ERK1/-2 MAPK in MRC-5 fibroblasts (Fig. 3-33A and B). The maximum increase in ERK1/-2 MAPK phosphorylation was seen within 5 minutes after exposure to ET-1. The effect of 100 nM ET-1 was somewhat stronger than that of oxotremorine (10 µM, a maximally effective concentration) (Fig. 3-33B). There was no change in the level of total ERK under these conditions, indicating that the increase in phosphorylation of ERK was due to an increase in the level of ERK activation. Like the other functional responses, ET-1-mediated activation of ERK1/-2 MAPK is predominately mediated by ET-A receptors, which is in line with the previous findings of Gallelli *et al.,* (2005).

4.5.2. Effects of pertussis toxin on ET-1-induced activation of ERK1/-2 MAPK and collagen synthesis stimulation

As outlined in the introduction, various observations indicate, that MAP kinases may be stimulated by activation of G_i and G_q protein-coupled receptors through different signal pathways (Marshall, 1994) and that ERK activation may be triggered by stimulation of G_i-coupled receptors (Touhara *et al.,* 1995; van Biesen *et al.,* 1995; Della Rocca *et al.,* 1997; Yahiaoui *et al.,* 2006). GPCRs downstream pathways have been divided into two sub-groups, PTX-sensitive (coupled to $G_{i/o}$) or PTX-insensitive (coupled to $G_{q/11}$, G_s, or $G_{12/13}$) (Albert *et al.,* 2002; Pierce *et al.,* 2002). ET-1 mediates its biological actions through the activation of membrane G protein coupled ET-A and ET-B receptors (Arai *et al.,* 1990; Sakurai *et al.,* 1990), which may couple to several G_α subunits, including $G_{i/o}$, $G_{q/11}$, $G_{12/13}$, and G_s (Takagi

et al., 1995; Cadwallader *et al.,* 1997; Arai *et al.,* 2003). Moreover, it has been shown that ET-1-mediated responses, including MAPK activation, are mediated by $G_{\alpha q}$, $G_{\alpha i}$, and $G_{\beta\gamma}$ (Miyauchi *et al.,* 1999).

In the present study, the contribution of G_i signalling to the activation of ERK pathway in MRC-5 fibroblasts by ET-1 and muscarinic receptor oxotremorine was assessed by using pertussis toxin (PTX).

In confirmation of previous observations demonstrating that muscarinic receptors in human lung fibroblasts are G_i coupled (Matthiesen *et al.*, 2006; Haag *et al.*, 2008a), PTX prevented oxotremorine-induced ERK activation (Fig. 3-34), whereas ERK1/-2 activation induced by ET-1 was only partially inhibited by PTX (Fig. 3-34). Since a single receptor can couple to different G proteins, these results suggest that ET-1 through the ET-A receptor subtype, may activate ERK in part via PTX-sensitive G_i, but to a large extent also via PTX-insensitive G-protein, possible G_q or $G_{11/12}$. This is in line with findings in rat myometerial cells, where ET-1-mediated stimulation of MAPK signalling through activation of ET-A receptor was shown to occur via a combination of PTX-sensitive and PTX-insensitive mechanisms (Robin *et al.,* 2002). The dual mechanism of activation may account for the stronger signal induced by ET-1 compared to oxotremorine. The observation that PTX caused a reduction in the basal phospho-ERK1/-2 levels suggests a background activation of G_i-mediated effects, possibly caused by paracrine mediators released by the fibroblasts themselves.

Furthermore, in correspondence with the observation on ERK activation, the stimulation of collagen synthesis induced by muscarinic agonist was prevented by pertussis toxin, whereas that caused by ET-1 was only partially reduced (as discussed previously).

4.5.3. Role of ERK1/-2 MAPK in ET-1-mediated stimulation of collagen synthesis

ET receptors mediate their effects by generation of the second messengers and subsequent activation of target proteins including ERK1/-2 (Kasuya *et al.,* 1994) which subsequently result in activation of mitogenic and survival signals (Rozengurt, 2007). The MAP kinase ERK pathway regulates a number of essential cellular

functions (Kolch *et al.,* 2005). Thus, ERK signalling has been demonstrated to be crucially involved in the proliferation and differentiation of human lung fibroblasts into myofibroblasts (Gallelli *et al.,* 2005; Vancheri *et al.,* 2005; Matthiesen *et al.,* 2007; Caraci *et al.,* 2008). Moreover, ERK activation has also been shown to be crucial for stimulatory effects on collagen synthesis induced by various stimuli, for example also for the effects of ET-1 in rat cardiac fibroblasts (Rhaleb *et al.,* 2001), TGF-β and muscarinic receptors in human lung fibroblasts (Caraci *et al.*, 2008; Haag *et al.*, 2008a) or vascular endothelial growth factor (VEGF) in human mesangial cells (Amemiya *et al.,* 1999).

The present study demonstrated, that in human lung fibroblasts ET-1 can stimulate collagen synthesis and activate ERK1/-2 MAPK via ET-A receptor. Therefore, it was investigated whether the MAPK pathways are involved in the ET-1-induced stimulation of collagen synthesis by interaction studies using PD98059, a specific inhibitor for MAPK-activating enzyme (MEK) (Alessi *et al.,* 1995; Dudley *et al.,* 1995). ET-1-induced stimulation of collagen synthesis was completely blocked by this ERK-MAPK inhibitor, indicating that the ERK MAPK pathway is crucially involved in the collagen synthesis response of human lung fibroblasts to ET-1. Noteworthy, ET-1 stimulated both ERK activity and collagen synthesis via the same ET receptor subtype, the ET-A receptor as determined by the effect of the ET-A antagonist BQ-123, which prevented the stimulation of collagen synthesis and activation of ERK1/-2. Interestingly, ET-1-mediated ERK-MAPK activation has also been shown to be responsible for an increased expression of several other matrix-associated genes and the stimulation of production of other ECM components in lung fibroblasts, including connective tissue growth factor, an important profibrotic protein that induces synthesis of collagen (Shi-Wen *et al.,* 2004; Xu *et al.,* 2004). Since collagen production is one of the most recognized indicators of fibrosis (Fine *et al.,* 1987; Hafizi *et al.,* 2004), these results further suggest that there may be a potential therapeutic advantage in using MEK/ERK kinase inhibitors or ET-A receptor antagonists to alleviate lung fibrosis. A potential role of ET-A receptor and ERK1/-2 activation in ET-1-induced lung fibrosis is supported by the observations that the ET-A receptor antagonist and the ERK-MAPK inhibitor prevented the synthesis of collagen induced by ET-1. Furthermore, a pathophysiologic relevance of an ET-A receptor mediated activation of ERK1/-2 MAPK in human lung fibroblasts is also

suggested by other studies demonstrating that ET-A receptors and ERK1/-2 mediate the ET-1-induced proliferation and differentiation into myofibroblasts (Gallelli *et al.,* 2005).

4.5.4. Role of Rho kinase in ET-1-mediated stimulation of collagen synthesis

As discussed, ET-1-induced stimulation of collagen synthesis in MRC-5 fibroblasts is mediated through the classical MEK-ERK MAPK cascade. As the low molecular weight G protein, Rho, is one effector of G_i, G_q and $G_{12/13}$, which regulates ERK1/-2 activation via its target Rho kinase (Coleman *et al.,* 2004), it was investigated whether Rho-kinase is involved in the ET-1-stimulated collagen synthesis. It has been shown that Rho kinase pathways can mediate effects of ET-1 in a variety of cell types (Rodriguez-Vita *et al.,* 2005; Tokuda *et al.,* 2007; Lima *et al.,* 2011; Seyler *et al.,* 2012; Undem *et al.,* 2012).

In the present study, inhibition of Rho kinase by Y-27632 partially, but significantly attenuated the levels of phosphorylated ERK induced by ET-1 (Fig.3-35), suggesting that Rho kinase is involved in the activation of ERK by ET-1.

Furthermore, the stimulatory effect of ET-1 on the synthesis of collagen was also partially reduced by Y-27632. Interestingly, although inhibition of Rho kinase partially inhibited ET-1-induced activation of ERK1/-2 (Fig 3-39), it had no significant effect on the ability of ET-1 to stimulate [^{3}H]-thymidine incorporation (Fig 3-24). These data indicate that in human lung fibroblasts, different signaling systems mediate the different effects of ET-A receptors.

5. Conclusion

In conclusion, human lung fibroblasts are endowed with all elements necessary to build a functional autocrine/paracrine endothelinergic system which appears to be involved in the regulation pro-fibrotic features. ET-1 is the predominant ET isoform in human lung fibroblasts, and its expression appears to be highly regulated at transcriptional level, suggesting that ET-1 expression may rapidly respond to physiological or pathological changes, as well as pharmacological interventions. The expression of ET-1 gene is under strong inhibitory control of short-living regulatory peptide, not yet identified. ET-1 may in interaction with other mediators such as TGF-β as well as the adrenergic and cholinergic system, drive pro-fibrotic features. Thus, ET-1 is highly up-regulated by TGF-β and via muscarinic cholinergic receptors and down-regulated via β_2-adrenoceptors. ET-1 can mediate through ET-A receptors stimulatory effects on proliferation and collagen synthesis. In fact, there is no functional significance of ET-B receptors obtained although the expression of ET-B receptor was demonstrated, both at mRNA and protein level.

6. Summary

Airway remodeling and fibrotic alterations are pathological key events determining the long-term outcome of chronic inflammatory and obstructive airway diseases such as lung fibrosis, bronchial asthma or COPD. In fact, these pathological conditions have a common tendency toward enhanced cellular activities mainly increased growth of connective tissue cells and excessive synthesis and secretion of matrix proteins. Lung fibroblasts are the most abundant cell type in connective tissue that produce and maintain extracellular matrix (ECM). They are essential in tissue homeostasis and fibrotic remodeling processes and thus play a key role in pathophysiologic events of airway remodelling. Hyper-activation of airway fibroblasts may result as a consequence of over expression of cytokines and/or growth factors like transforming growth factor (TGF-β) and endothelin-1 (ET-1) resulting from inadequate control of inflammation. The excessive ECM deposition and scar formation ultimately lead to functional impairment of the lung.

Since endothelins (ETs), of which three isoforms (ET-1, ET-2 and ET-3) exist, can exert a number of effects which could promote remodeling processes, the present study aimed to characterize the endothelinergic system in human lung fibroblasts using the MRC-5 human lung fibroblast cell line and primary human lung fibroblasts in culture.

The expression of ETs and their receptors was analyzed at the mRNA level by quantitative real time PCR (qPCR) and at the peptide level by immune blot for the ET-A and ET-B receptor and by ELISA for ET-1. Functional effects of ET-1 on cell proliferation were quantified using a [^{3}H]-thymidine incorporation assay and cell counts, those on collagen synthesis using a [^{3}H]-proline incorporation assay, the QuickZyme assay and qPCR for collagen-1 α1 mRNA. In addition ET-1-induced changes on phenotype were detected by measuring α-smooth muscle actin (α-SMA) at the protein level.

This study demonstrates that human lung fibroblasts clearly expressed ppET-1 and ppET-2 with the most dominantly expressed isoform being the ppET-1 followed by the ppET-2. On the other hand, no transcript was detected for ppET-3. Moreover, ET-A and ET-B receptors were also expressed in human lung fibroblasts. After the expression of ET-1 and its receptors in human lung fibroblasts was demonstrated,

mechanism involved in their regulation as well as their functional role in mediating proliferation and collagen synthesis were studied.

In summary, the following results were obtained:

1) The expression of ppET-1 and the release of ET-1 in human lung fibroblasts were significantly up-regulated by TGF-β. TGF-β-induced up-regulation of ppET-1 was augmented by concomitant ET-A receptor activation. Moreover, the stimulatory effect of TGF-β on the expression of ET isoforms is confined to ET-1.
   a) The expression of ppET-1 is highly regulated at the transcription level, and is under strong inhibitory control of not yet identified short acting living inhibitory proteins which also counteract TGF-β-induced up-regulation of ppET-1.
   b) The stimulatory effect of TGF-β did not show any fatigue during prolonged exposure, indicating that pathological conditions with prolonged elevation of TGF-β might be accompanied by a sustained up-regulation of ET-1 expression.
   c) TGF-β induced an increase in ppET-1 gene transcription through its canonical Smad-pathway, but ERK MAPK and Rho kinase signalling pathways are not involved.
   d) ET-B receptor was markedly down-regulated by TGF-β, whereas ET-A receptor was only marginally affected after prolonged exposure to TGF-β.
2) ET-1 expression is regulated by stimulatory muscarinic receptors and inhibitory β_2-adrenoceptors.
   a) The stimulatory effect of muscarinic receptors on ppET-1 expression was opposed by concomitant activation of β_2-adrenoceptors and by blocking of ET receptors.
   b) The inhibitory effect of β_2-adrenoceptors on ppET-1 expression appears to be mediated via PKA, as it was mimicked by direct activation of PKA.
   c) The stimulatory effect of sub-maximally effective concentration of TGF-β on ppET-1 expression was opposed by β_2-adrenoceptor-PKA pathway. However, excessive exposure to TGF-β results in loss of β-adrenoceptor expression and function of its down-stream signaling.

   Given that ET-1 can promote pro-fibrotic events in human lung fibroblasts, inhibition of ET-1 expression could contribute to long-term beneficial effects of

long-acting β_2-adrenoceptors agonists such as olodaterol and long-acting muscarinic antagonists like tiotropium.

3) ET-1-induced stimulation of proliferation of human lung fibroblasts was shown to be mediated via ET-A receptors, and Rho kinase pathway was not involved in this effect.
4) ET-1 induced differentiation of human lung fibroblasts into myofibroblasts, but was not involved in TGF-β-mediated human lung fibroblasts differentiation into myofibroblasts.
5) ET-1-induced stimulation of collagen synthesis in human lung fibroblasts was mediated via ET-A receptors and involved activation of the ERK-MAPK pathway partially via pertussis toxin-sensitive G proteins. The activation of collagen synthesis is at least in part caused by increase transcription as ET-1 induced up-regulation of COL I-α1 mRNA expression.
6) Interaction experiments with ET receptor antagonists indicate that activation of ET-A receptor contributed to the TGF-β-induced stimulation of collagen synthesis. Thus autocrine positive feedback indicates that the up-regulation of ET-1 gene by TGF-β is of functional significance and contributes to the pro-fibrotic effects of TGF-β.
7) Blockade of ET receptors also attenuated the muscarinic receptor-mediated stimulation of collagen synthesis, indicating that up-regulation of ET-1 gene by muscarinic receptor is of functional significance and contributes to the pro-fibrotic effects of cholinergic stimuli.

In conclusion, human lung fibroblasts express a functional autocrine/paracrine endothelinergic system that appears to be involved in the regulation pro-fibrotic features. ET-1 is the predominant ET isoform in human lung fibroblasts, which may in interaction with other mediators such as TGF-β as well as the adrenergic and cholinergic system, regulate pro-fibrotic features. Thus, ET-1 is highly up-regulated by TGF-β and via muscarinic cholinergic receptors and down-regulated via β_2-adrenoceptors. ET-1 can mediate through ET-A receptors stimulatory effects on proliferation and collagen synthesis. In fact, there is no functional significance of ET-B receptors obtained although the expression of ET-B receptor was demonstrated, both at mRNA and protein level.

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8. Publications and published congress abstracts

I. Publications

1. **Ahmedat AS**, Warnken M, Seemann WK, Mohr K, Kostenis E, Juergens UR, Racké K. Pro-fibrotic processes in human lung fibroblasts are driven by an autocrine/paracrine endothelinergic system. Br J Pharmacol. 2013; 168(2):471-87.
2. **Ahmedat AS**, Warnken M, Juergens UR, Paul Pieper M, Racké K. β_2-Adrenoceptors and muscarinic receptors mediate opposing effects on endothelin-1 expression in human lung fibroblasts. Eur J Pharmacol. 2012; 691(1-3):218-24.
3. 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(2):133-145.

II. Published conference abstracts

1. Warnken Mareille, **Ahmedat Ahmedat**, Reitzenstein Ulrich, Holtin Stefan M., Mayer Peter, Juergens Uwe R, Racké Kurt. (2009). Differential effects of TGF-β on expression of prepro-endothelin-1 and phenotype markers in human alveolar type lung (A 549) and bronchial (H 292) epithelial cells. Proceedings of the British Pharmacological Society at http://www.pA2online.org/abstracts/ Vol7 Issue2abst014P.

2. **Ahmedat S. Ahmedat**, Mareille Warnken, Meinolf Stöber, Uwe R. Juergens, Kurt Racké. Characterization of endothelinergic mechanisms in human lung fibroblasts. Am J Respir Crit Care Med 18; 2010:A3538.

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9. Acknowledgment

This thesis would not have been possible to exist without the extraordinary guidance and the tremendous help of several individuals who in one way or another contributed and extended their valuable assistance in the preparation and completion of this study.

First and foremost, I would like to express my grateful appreciation and sincere thanks to my supervisor ***Prof. Dr. Kurt Racké****, for being a faithful mentor, for his timely advice, his kind and patient instructions, motivation, continuous support, encouragement and for offering me the opportunity to do a PhD in his group. I want also to thank you for the time you spent to read the PhD thesis and for the valuable comments you made to it.*

I would like further to express my deepest gratitude to my co-supervisor ***Prof. Dr. Klaus Mohr****, for his acceptance to be the co-supervisor of my thesis, for his time, brilliant comments, suggestions and evaluation of my thesis.*

My appreciation is also extended to the other member of doctoral committee, ***Prof. Dr. Alf Lamprecht and Prof. Dr. Arne Lützen.*** *I would like deeply to thank them for their willingness to act as co-examiner, their time spending to read and evaluate the thesis, their insightful comments, and suggestions.*

My gratitude and warmly thanks to the former and current colleague lab members for their valuable contribution and assistance, and for providing a pleasant working environment where I enjoyed doing my research, and to all members of the Pharmacology and Toxicology institute for their support during my PhD in the department.

I would like to convey my warm thanks to ***Mrs. Rita Fuhrmann*** *for the kind help and for her excellent technical assistance.*

I wish to thank the Libyan government for financial support.

Last but not least a heartfelt thank goes to my dear parents, for their motivational support and encouragement throughout my life, siblings and friends who have supported me at all stages. My special thanks go to my wife and my kids for their help, patience, encouragement and invaluable support.