DISSERTATION

The structural and functional activation of Endothelin-1 type A receptors (ET_AR)

Die strukturelle und funktionelle Aktivierung des Endothelin-1-Typ-A-Rezeptors (ET_AR)

zur Erlangung des akademischen Grades
Doctor rerum medicinalium (Dr. rer. medic.)

vorgelegt der Medizinischen Fakultät

Charité – Universitätsmedizin Berlin

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Datum der Promotion: 30.06.2024

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3T3	Swiss-3-day transfer inoculum of 3 x 10^5 fibroblast cell line
α_{1A}-AR	Alpha-$_{1A}$-adrenoreceptor
α_{1B}-AR	Alpha-$_{1B}$-adrenoreceptor
α_{1D}-AR	Alpha-$_{1D}$-adrenoreceptor
α-SMA	Alpha smooth muscle actin
µg/mL	Microgram per milliliter
µL	Microliter
µm	Micrometer
µM	Micromolar
AA	Amino Acids
AAbs	Autoantibodies
ACA	Anti-centromere antibodies
ANA	Anti-nuclear antibodies
Anti-RNAP	Anti-ribonucleic acid polymerase antibodies
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
AT_1R	Angiotensin II type I receptor
AT_2R	Angiotensin II type 2 receptor
ATA	Anti-topoisomerase I antibodies
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CCL 18	Chemokine (c-c motif) ligand 18
cDNA	Complementary deoxyribonucleic acid
CHO	Chinese hamster ovary cell line
CHO-KI	Chinese hamster ovary cells lacking the gene for glycine biosynthesis
CMV	Cytomegalovirus
CO_2	Carbon dioxide
COS	CV-1 in origin, and carrying the SV40 genetic material
Cter	Carboxyl terminus
Cys	Cysteine
DAG	Diacylglycerol
ddH_20	Distilled water
del	Deletion
dSSc	Diffused cutaneous systemic sclerosis
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EC	Endothelial cells
ECD	Extracellular domain
ECE	Endothelin converting enzyme
ECL	Extracellular loop

EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
EPAC	Exchange protein of activated cAMP
ER	Endoplasmic reticulum
ERA	Endothelin receptor antagonist
ERK 1/2	Extracellular signal-related kinase 1/2
ET-1	Endothelin-1
ET-2	Endothelin-2
ET-3	Endothelin-3
ET_AR	Endothelin type A receptor
ET_BR	Endothelin type B receptor
Ets-1	E26 transformation-specific transcription factor-1
FCS	Fetal calf serum
Glu	Glutamic acid
GPCR	G-protein coupled receptor
GTP	Guanosine triphosphate
HCl	Hydrogen chloride
HEK 293 T	Human embryonic kidney 293 T cell line
HeLa	Henrietta Lacks cell line
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMEC	Human microvascular endothelial cells
hr	Hour
IBMX	3-isobutyl-1-methylxanthine
ICD	Intracellular domain
IFN-γ	Interferon-gamma
IgG	Immunoglobulin
IL-8	Interleukin-8
IP_3	Inositol 1,4,5-triphosphate
kbp	Kilobase pair
KCl	Potassium chloride
KH_2PO_4	Monopotassium phosphate
KLD	Kinase-Ligase-*Dpn*I
LB	Lysogeny broth
lSSc	Localised systemic sclerosis
Lys	Lysine
M	Molar
m/v	Mass per volume
MAPK	Mitogen-activated protein kinase
Met	Methionine
mg/mL	Milligram per milliliter
Mg^{2+}	Magnesium
min	Minutes
mL	Milliliter
MLCK	Myosin light chain kinase
mM	Millimolar
MuLV	Murine leukaemia virus
Na_2HPO_4	Disodium phosphate

NaCl	Sodium chloride
NFAT	Nuclear factor of activated T-cell
ng	Nanogram
ng/µL	Nanogram per microliter
NO	Nitric oxide
Nter	Amino terminus
PAH	Pulmonary arterial hypertension
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
PKA	Protein kinase A
PKC	Protein kinase C
PLB	Passive lysis buffer
PLC	Phospholipase C
RhoA	Rat sarcoma virus protein homologue A
RNAse	Ribonuclease
ROCK	Rho-associated coiled kinase
rpm	Revolutions per minute
s	Seconds
Ser	Serine
SOC	Super optimal catabolite
SPS	Signal peptide sequence
SRC	Scleroderma renal crisis
SRE	Serum response element
SRF	Serum response factor
SSc	Systemic sclerosis
TGF-β	Transforming growth factor–beta
Thr	Threonine
TMD	Transmembrane domain
Tyr	Tyrosine
v/v	Volume per volume
VCAM-1	Vascular adhesion molecule-1
VSMC	Vascular smooth muscle cell
WT	Wild-type

Abstract

Autoantibodies against the endothelin type A receptor (ET_AR) (ET_AR-AAbs), a G protein-coupled receptor, are recognizable players in vasculopathies, especially systemic sclerosis. Activation of ET_AR by its natural ligand, endothelin-1 (ET-1) can be pathogenic. In this case, similar clinical features as in ET_AR-AAbs activation are observed. However, the binding domain of both ligands is unknown. Given the accessibility and uniqueness of the extracellular amino terminus of ET_AR (ET_AR-Nter), it was hypothesized that both ligands activate different domains of ET_AR-Nter to trigger distinct signaling dynamics. Therefore, the role of ET_AR-Nter in modulating differences in ET-1 and an ET_AR-AAbs-mediated signaling mechanism in a human embryonic kidney cell line was investigated. Three different parts of ET_AR-Nter were sequentially deleted by site-directed mutagenesis namely, amino acid (AA) 46-65, 26-65, and 2-65. The relative membrane expression of ET_AR was determined using the NanoGlo® HiBiT extracellular detection assay. Transfected cells with or without a luciferase reporter were independently stimulated with different doses of ET-1 and ET_AR-AAbs to determine their respective effect on G-proteins and extracellular signal-regulated protein kinase 1/2 (ERK 1/2) activation. The effect of ET_AR-Nter on ET_AR-AAbs-mediated cyclic adenosine monophosphate (cAMP) production was also assessed using enzyme-linked immunosorbent assay. ET_AR-AAbs and ET-1 activated $G_{q/11}$, $G_{12/13}$ and ERK 1/2, but ET_AR-AAbs induced a stronger production of cAMP than ET-1. The loss of AA 2-25 increased relative membrane expression of ET_AR but decreased ET-1 and ET_AR-AAbs-mediated intracellular activation. Loss of AA 46-65 and 26-45 had no effect on ligand-mediated activation of $G_{q/11}$ and ERK 1/2. Rather, ET-1 induced $G_{12/13}$ activation via AA 26-45, while ET_AR-AAbs affected $G_{q/11}$ and $G_{12/13}$ activation via AA 2-25 and 46-65 respectively. ET_AR-AAbs also reduced cAMP levels due to AA 26-45. This shows that ET_AR-AAbs are agonistic but different parts of ET_AR-Nter influence signaling selectivity. The results demonstrate the need for structural modeling of antibody-ET_AR to identify binding domains useful for drug selectivity in ET_AR-AAbs-mediated vasculopathies such as systemic sclerosis.

Abstrakt

Autoantikörper gegen den Endothelin-Typ-A-Rezeptor (ET_AR) (ET_AR-AAbs), einen G-Protein-gekoppelten Rezeptor, spielen bei Vaskulopathien, insbesondere bei systemischer Sklerose, eine erkennbare Rolle. Die Aktivierung von ET_AR durch seinen natürlichen Liganden, Endothelin-1 (ET-1), kann pathogen sein. In diesem Fall werden ähnliche klinische Merkmale wie bei der Aktivierung von ET_AR-AAbs beobachtet. Der Bindungsbereich beider Liganden ist jedoch unbekannt. Angesichts der Zugänglichkeit und Einzigartigkeit des extrazellulären Aminoterminus von ET_AR (ET_AR-Nter) wurde die Hypothese aufgestellt, dass beide Liganden unterschiedliche Domänen von ET_AR-Nter aktivieren, um unterschiedliche Signaldynamiken auszulösen. Daher wurde die Rolle von ET_AR-Nter bei der Modulation von Unterschieden in ET-1 und einem ET_AR-AAbs-vermittelten Signalmechanismus in einer menschlichen embryonalen Nierenzelllinie untersucht. Drei verschiedene Teile von ET_AR-Nter wurden nacheinander durch ortsgerichtete Mutagenese entfernt, nämlich die Aminosäuren (AA) 46-65, 26-65 und 2-65. Die relative Membranexpression von ET_AR wurde mit dem extrazellulären NanoGlo® HiBiT-Nachweisassay bestimmt. Transfizierte Zellen mit oder ohne Luciferase-Reporter wurden unabhängig voneinander mit verschiedenen Dosen von ET-1 und ET_AR-AAbs stimuliert, um ihre jeweilige Wirkung auf G-Proteine und die Aktivierung der extrazellulären signalgesteuerten Proteinkinase 1/2 (ERK 1/2) zu bestimmen. Die Wirkung von ETAR-Nter auf die durch ETAR-AAbs vermittelte Produktion von zyklischem Adenosinmonophosphat (cAMP) wurde ebenfalls mit einem Enzymimmunoassay untersucht. ET_AR-AAbs und ET-1 aktivierten $G_{q/11}$, $G_{12/13}$ und ERK 1/2, aber ET_AR-AAbs induzierte eine stärkere Produktion von cAMP als ET-1. Der Verlust von AA 2-25 erhöhte die relative Membranexpression von ET_AR, verringerte jedoch die ET-1- und ET_AR-AAbs-vermittelte intrazelluläre Aktivierung. Der Verlust der AA 46-65 und 26-45 hatte keine Auswirkung auf die Liganden-vermittelte Aktivierung von $G_{q/11}$ und ERK 1/2. Vielmehr induzierte ET-1 die $G_{12/13}$-Aktivierung über AA 26-45, während ET_AR-AAbs die $G_{q/11}$- und $G_{12/13}$-Aktivierung über AA 2-25 bzw. 46-65 beeinflussten. ET_AR-AAbs verringerten auch den cAMP-Spiegel durch AA 26-45. Dies zeigt, dass ET_AR-AAbs agonistisch sind, aber verschiedene Teile von ETAR-Nter die Signalselektivität beeinflussen. Die Ergebnisse zeigen, dass eine Strukturmodellierung von Antikörper-ET_AR notwendig ist, um Bindungsbereiche zu identifizieren, die für die Arzneimittelselektivität bei ET_AR-AAbs-vermittelten Vaskulopathien wie der systemischen Sklerose nützlich sind.

1 Introduction

1.1 Clinical Background

Systemic sclerosis (SSc) is an uncommon autoimmune connective tissue disease that poses major health challenges worldwide (1). SSc is classified into two forms comprising the more restrictive localized cutaneous SSc (lSSc) and diffuse cutaneous SSc (dSSc) (1). Diffuse SSc is usually accompanied by multi-organ involvement (1) and will be the subject of this work. The pathogenesis of SSc is complex but typically has three main features: extensive fibrosis, a vascular crisis and a defective autoimmune system (1). Remarkably, most SSc patients with the dSSc subtype are prone to and die from cardiovascular and pulmonary complications (1). Despite the increased mortality and morbidity of SSc, available treatment approaches only focus on complications (2). The lack of a specific therapeutic strategy for SSc is attributed to the complex pathophysiology that creates an interface between immunological and non-immunological activation (1).

However, extensive evidence suggests that initial vascular damages initiate the SSc disease process (3, 4). This vascular damage from genetic and environmental sources disrupts the structural integrity and function of the endothelium (3). The activated endothelium subsequently releases pathological factors including the vasoactive peptide, endothelin-1 (ET-1) and proinflammatory and profibrogenic cytokines, which mediates the vascular remodeling and extracellular matrix deposition observed in SSc (3-5). As such the detection of high serum/plasma levels of ET-1 and the expression of the cognate receptor, endothelin type A receptor (ET_AR) has therefore been associated with SSc patients with complications such as pulmonary arterial hypertension (PAH) and scleroderma renal crisis (SRC) (5, 6). Mechanistic studies have further elucidated the pathogenicity of ET-1 to trigger the release of proinflammatory cytokines, fibrogenic and proliferative mediators of SSc (5, 6). This has warranted the recommendation for the use of endothelin receptor antagonists (ERA) in the SSc complications such as pulmonary arterial hypertension (PAH) (2).

In addition to the dysregulated vascular responses, the activated endothelium alters the immune system to evoke autoantibody (AAbs) production (3, 4). Although AAbs are naturally produced in healthy individuals, AAbs are somewhat indicated in the vascular crises seen in autoimmune diseases including SSc (7-9). Particularly, AAbs targeting the G protein-coupled receptor family

of receptors such as angiotensin II type 1 receptor and ET_AR are widely implicated in several autoimmune pathologies including SSc (7-9). In fact, in SSc patients, detectable ET_AR-AAbs are known to be associated with worse clinical complications and mortality (10). These SSc-ET_AR-AAbs stimulate the release of proinflammatory, proliferative and profibrogenic cytokine mediators of SSc similar to ET-1 (11, 12). Mechanistic studies have also demonstrated the potency of SSc-ET_AR-AAbs to induce the regulation of the proto-oncogene, E26 transformation-specific-1 transcription factor-1 (Ets-1), suggesting a role in vasculopathy (13). It is also speculated that ET_AR-AAbs enhance vasoconstrictive tendencies in SRC when co-stimulated together with ET-1 (14). Furthermore, passive transfer of ET_AR-AAbs from the sera of SSc patients with PAH also caused endothelial damage in mice (15), confirming pathogenicity. However, despite the proposed contribution of ET_AR-AAbs to SSc pathology, the binding domain and differences in signaling dynamics compared to ET-1 are unknown. Therefore, the role of ET_AR-AAbs in ET_AR signaling was examined in this work.

1.2 The Endothelin system

The endothelin system consists of three endothelin isoforms, ET-1, ET-2 and ET-3, which bind to two endothelin receptors, ET_AR and Endothelin type B receptor (ET_BR) (16, 17). The three Endothelin isoforms contain 21 amino acids (AA) but bind differently to the specific Endothelin receptors (16, 17). Pharmacological characterization studies show that the three isoforms bind to ET_AR, but ET-1 has been prominently studied (16).

1.2.1 Endothelin-1, synthesis and secretion

ET-1 is widely distributed in various tissues and cells, but is predominantly found in the endothelial cells (EC) (16). In EC, many factors, including hypoxia, oxidative stress and transcriptional regulation, mediate the synthesis of the bioactive ET-1 through a three-step process of proteolytic cleavages (16). At the beginning of the process, the transcribed endothelin gene encodes a 212 AA polypeptide called preproendothelin (16). Preproendothelin is further cleaved by a signal peptidase into a proendothelin, which is cleaved into big endothelin by a furin-like convertase (16). Finally, big endothelin is cleaved by the sub-specific endothelin converting enzyme (ECE) (16, 18). Later, secreted ET-1 is rapidly cleared mainly in the lungs by the clearance action of ET_BR and endocytosis (18, 19).

1.2.2 Endothelin-1, molecular structure

Since the discovery of ET-1, various attempts have been made to elucidate its molecular structure. The most recent X-ray crystal structure revealed the molecular structure consistent with previous cloning data (20). ET-1 uniquely contains 21 AA bound by a proximal amino terminus (Nter) (AA 1-15) and a distal carboxyl terminus (Cter) (AA 16-21) as seen in figure 1 (20-22).

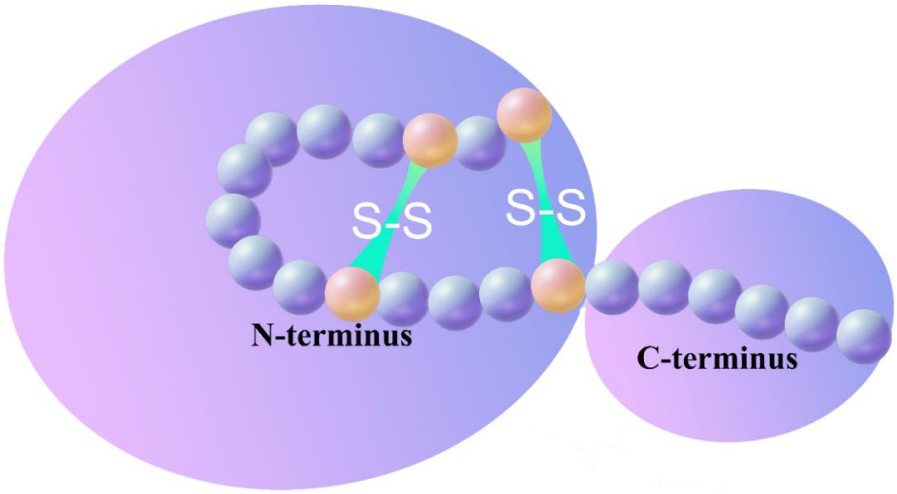

Figure 1: Molecular structure of endothelin-1 (ET-1).
A 21 amino acid (AA) based peptide with an amino terminus (Nter) from AA 1-15 and carboxyl terminus (Cter) from AA 16-21. Disulphide bridges are formed between cysteine residues (solid orange circles) 1 and 15 as well as Cys 3 and 10.

Two disulfide bridges hold the subunits that distinguish ET-1 from other homologues (20). Docking and ligand binding studies indicate that in addition to disulfide bridges, specific AA residues also affect binding efficiency (20, 21, 23). The crystal structure studies suggested a conformational switch between Nter and Cter affecting binding efficiency (20). In particular, the tryptophan at residue 21 of the Cter is involved in the activation of ET_BR (24). However, it is unclear whether specific AA residues of ET-1 affect ET_AR, as the crystal structure of the receptor has not yet been solved.

1.2.3 Endothelin receptors

The functional relevance of ET-1 is mediated by its action on two independent GPCR subfamily A, ET_AR and ET_BR (16, 17). Typical of GPCRs, endothelin receptors have a similar structural organization of seven transmembrane domains (TMD) connected by extracellular domains (ECD) and intracellular domains (ICD) (16, 17, 25). Moreover, ET_AR and ET_BR share a structural

homology in AA composition but differ substantially in the Nter domain (16, 24, 25). The Cter of both receptors couple to the heterotrimeric $G_{\alpha\beta\gamma}$ and β-arrestin, whose activation mediate a variety of intracellular mechanisms (26). Activation of both receptors produces a counterbalancing effect in maintaining the vascular tone (19, 27).

1.2.3.1 Endothelin type A receptor

The human ET_AR subtype is the most studied and well-characterized subtype of endothelin receptors (16). Naturally, the ET-1-induced action of ET_AR contributes to vascular homeostasis, the dysregulation of which is implicated in several vasculopathies (16, 27). Therefore, the expression and structure of ET_AR, which is predominantly located in vascular smooth muscle cells (VSMC) (16), is important for vascular pathophysiology and pharmacological inhibition (16). Although the crystal structure has not yet been found, cloning data show that the human ET_AR gene is mapped to chromosome 4 and has eight exons (16, 17). The ET_AR also bears the unique rhodopsin-like structure shared by to family A GPCR (16, 17, 25) , as seen in figure 2.

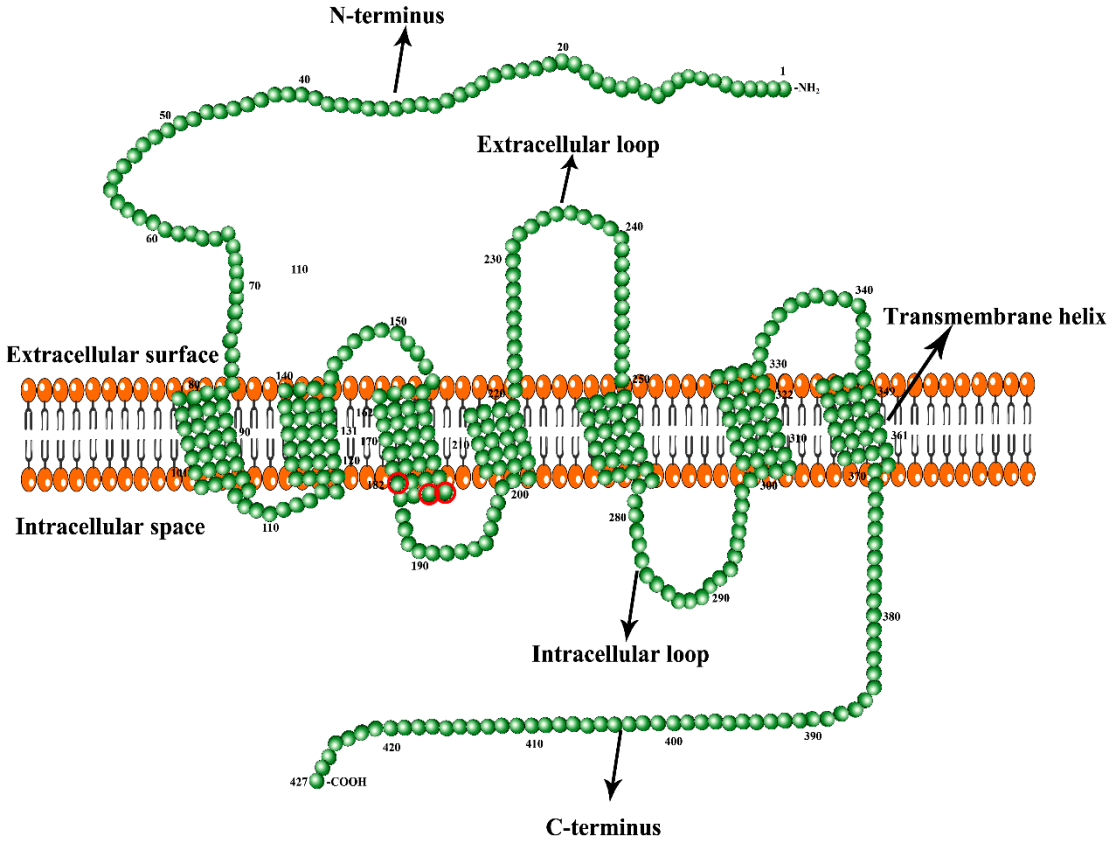

Figure 2: Structure of endothelin type A receptor (ET_AR).
A 427 polypeptide (amino acids shown as solid green circles) divided into an amino (N) terminus and extracellular loops, seven transmembrane helices and an intracellular domain consisting of; intracellular loops and a carboxyl terminus. Bilayer lipid plasma membrane is represented as solid orange circles.

The ET_AR polypeptide chain contains a 427 AA sequence forming seven helices, three ECD and ICD (16, 17). ET_AR-Nter has a long 80 AA sequence compared to ET_BR and other family A receptors (16, 24, 25). The Nter is glycosylated at asparagine (Asn) 29 and 62, but the role in membrane expression and ligand binding is not fully defined. The extracellular loops (ECLs) and TMD I-III and IV are thought to be involved in ligand interaction and serve as putative binding domains (25). Structural changes in the AA sequence of this putative binding domain therefore affect ligand interaction and receptor function. In particular, tyrosine (Tyr) 129 and aspartate (Asp) 147 mutations of TMD II have been shown to reduce ligand affinity and selectivity (28, 29). After the ligand interaction, the ET_AR changes its conformation, allowing the Cter to couple to independent G-protein subunits and β-arrestin (17, 26). Palmitoylation and ubiquitination of cysteine (Cys) residues of the Cter are thought to promote receptors coupling to G proteins to initiate specific intracellular signaling (30, 31). Later, signaling is deactivated by several processes

involving GPCR kinases, recruitment of β-arrestin and rapid internalization of ligand-receptor complexes (26, 32, 33).

1.2.3.2 Endothelin type B receptor

The ET_BR is a class A peptidergic receptor of the GPCR family with structural and functional relationship to the ET_AR (16, 17). Structurally, the ET_BR, which is prominent on EC located on chromosome 13 with seven exons shares about 60-70% structural homology with the ET_AR (25). Significantly, the extracellular Nter has the structural difference of bearing a short 64 AA and being glycosylated at Asn^{59} (34). The cleavable ET_BR-Nter (35) with a signal peptide sequence at AA 2-22 (36) is involved in ligand binding and receptor functionality (24, 37). Functionally, the ET-1-induced activation of ET_BR balances the vasoconstrictive function of ET_AR through vasodilation (19, 27). Given the relative restorative function of ET_BR, drug development is focused on targets against the pathogenic ET_AR, hence its signaling dynamics, a major focus in pharmacology.

1.3 Physiologic and pathophysiologic signaling of ET_AR

1.3.1 Physiologic signaling of ET_AR

ET-1-induced activation of ET_AR involves intricate downstream signaling mechanisms. As shown in figure 3, ET-1-mediated activation of ET_AR leads to conformational changes that result in coupling of specific G-protein subunits (16, 17). The ET_AR-G-protein coupling cascades into protein-protein interaction and gene regulation that is manifested in common cellular phenotypes of vasoconstriction, proliferation and remodeling (17, 38, 39).

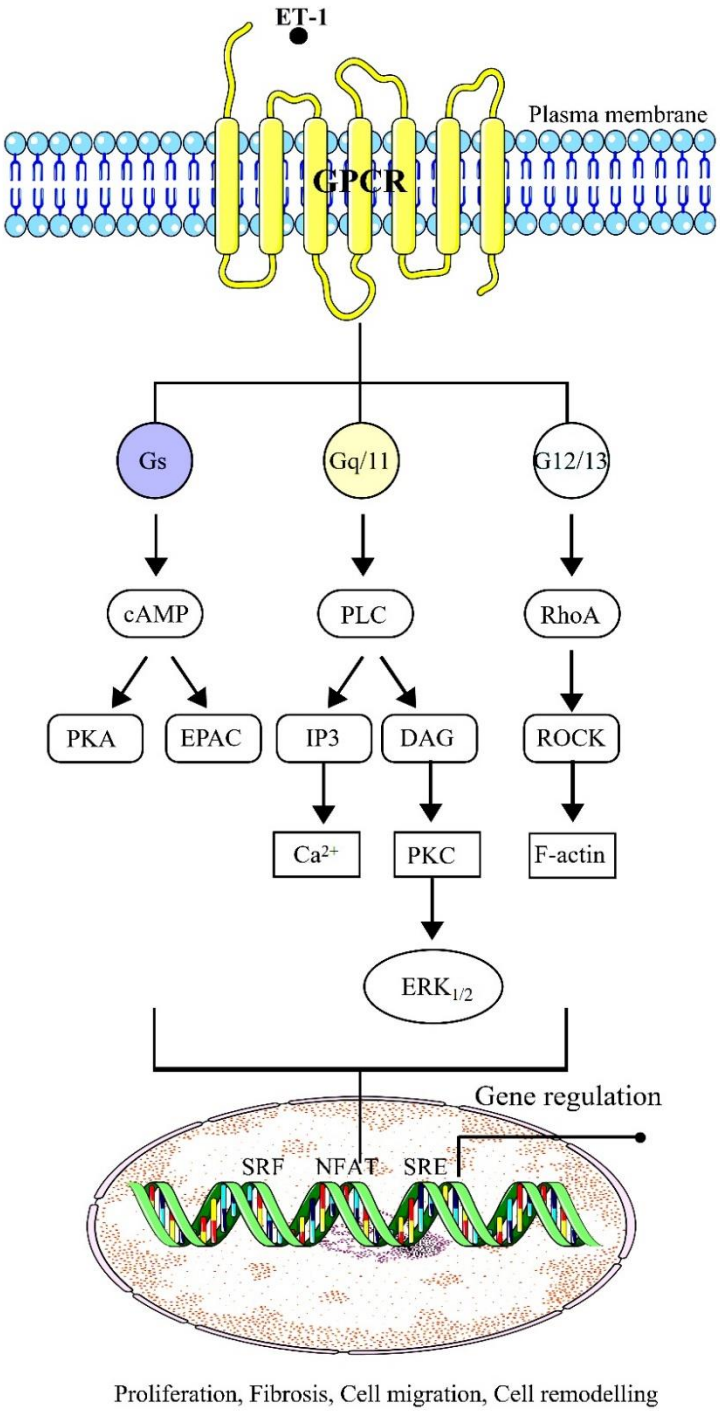

Figure 3: Endothelin-1 mediated ET_AR signaling mechanism.
ET-1, Endothelin-1; GPCR, G-protein coupled receptor; cAMP, cyclic adenosine monophosphate; PLC, Phospholipase C, RhoA, Rat sarcoma virus protein homologue A; PKA, Protein kinase A; EPAC, Exchange protein activated cAMP; DAG, Diacylglycerol, IP_3, Inositol 1,4,5-triphosphate; ROCK, Rho-associated coiled kinase; PKC, Protein kinase C; Ca^{2+}, Calcium^{2+}, ERK 1/2, Extracellular signal-related kinase; NFAT, Nuclear factor of activated T cell; SRF, Serum response factor; SRE, Serum response element.

Particularly, ET-1-induced production of [Ca^{2+}] via the $G_{q/11}$ coupling is the most researched due to its role in vasoconstriction (39). Data from pharmacology experiments with ECs, VSMCs, and animal models pre-treated with blockers demonstrate ET-1-induced elevation of intracellular

[Ca^{2+}] by activation of phospholipase C (PLC), which leads to the hydrolyses of phospholipids and, consequently, to the liberation of inositol-1, 4, 5-triphosphate (IP_3) and diacylglycerol (DAG) (17). Although ET-1 induces the influx of [Ca^{2+}] from extracellular stores, IP_3-induced intracellular accumulation of [Ca^{2+}] is observed to explain the sustained vasoconstriction typical of ET-1-mediated ET_AR signaling (17). This phenomenon has been further proven in a recent study where deletion of IP_3-receptors in a mouse model without compensatory secretion from extracellular stores diminished sustained vasoconstriction (40). Although intracellular [Ca^{2+}] can be elevated by IP_3-independent mechanisms such as the perinuclear space and by activation of DAG (17), activation of protein kinase C (PKC) is regulated by DAG, the inhibition of which attenuates intracellular [Ca^{2+}] accumulation (17) thereby redirecting sustained vasoconstriction on IP_3-mediated secretion. Increased intracellular [Ca^{2+}] target multiple downstream mediators, including myosin light chain kinase (MLCK) and calcineurin-induced activation of Nuclear Factor of Activated T-cells (NFAT), which promote vasoconstriction, apoptosis, and immune cell activation (41). ET-1-induced activation of ET_AR also couples to $G_{12/13}$ which is also recognized in vascular remodeling and contraction (38). Although the downstream mechanisms are still being examined, *in vitro* studies in different cell lines, such as Chinese hamster ovary (CHO) cells, cardiomyocytes and aortic smooth muscle cells, overexpressing $G_{12/13}$ mutant or inhibitory experiments have shown ET_AR-dependent coupling of $G_{12/13}$ and subsequent activation of rat sarcoma virus protein homologue A (RhoA) (42). Pharmacological studies further show that Rho-associated coiled kinase (ROCK) and MLCK activation leads to cytoskeletal remodeling (42, 43). Again, ET_AR-induced activation of RhoA also regulates the formation of stress fiber by regulating the transcription of serum response factor (SRF) (44). To this end, SRF has been used extensively as an indirect determinant of $G_{12/13}$ activation (45, 46).

Besides vasoconstriction, ET_AR signaling also causes proliferation via independent G-protein pathways. It is known that many independent downstream effectors of ET_AR converge to elicit differential mitogen-activated protein kinase (MAPK) signaling and gene-regulation of proto-oncogenes (17, 38). For example, ET-1 coupling of $G_{q/11}$ activates PKC which in turn increases MAPK activity (17, 38). ET-1-induced ERK 1/2 activation is also regulated by the Ras-Raf proto-oncogene and nuclear transcription factor, serum response element (SRE) (17, 38). Furthermore, ET-1-induced $G_{12/13}$ coupling also promotes ERK 1/2 and the c-Jun amino2-terminal kinase, thus contributing significantly to vascular proliferation and remodeling (17, 38).

ET-1 also mediates the production of cAMP but the pathways are not fully defined. Studies with different cell lines and tissues demonstrate ET_AR signaling of cyclic adenosine monophosphate (cAMP) via G_s-dependent and independent mechanisms (47, 48). For example, ET-1 induces cAMP production by activating the cyclooxygenase and $G_{\beta\gamma}$ pathways as well as protein kinase A (PKA), an effector of cAMP (48, 49). Produced cAMP then targets many intracellular effectors, including protein kinase C (PKC) and exchange protein of activated cAMP (EPAC), whose role in vascular and autoimmune diseases is receiving much attention recently (50, 51).

1.3.2 Pathophysiology of ET_AR signaling

The endothelin system is one of the key systems involved in the regulation of the vascular tone. The endothelin system is therefore significantly involved in the vascular hemodynamics of the renal, cardiovascular and immune systems (16, 27, 52). ET-1 secreted by the EC mainly acts on smooth muscle cells through the production of Ca^{2+} and contractile proteins to regulate vasoconstriction (19, 27). ET-1 thus controls the contractility of cardiac muscles (52) and also regulates glomerular filtration rate and salt balance in the kidneys (53). Elevated ET-1 therefore increases renal vascular resistance and thereby reduces blood flow rate, as has been shown in isolated or perfused kidneys (53). In addition, ET-1 is also involved in neuromodulation, synthesis of adrenocortex, and modulation of the immune system, but these roles are still under investigation (16).

Aberrant regulation of endothelin signaling therefore contributes to the pathophysiology of several diseases, including pulmonary hypertension, chronic heart failure, renal failure and SSc (52, 54). For example, PKC-mediated activation of the MAPK pathway, proto-oncogenes and Ca^{2+}-induced sustained vasoconstriction is suggested to underlie the pathophysiology of atherosclerosis, hypertension, kidney damage and PAH (52, 54). In SSc, ET-1 acting via ET_AR produces effects leading to inflammation, cell migration, cell remodeling and proliferation in both immune and non-immune cells (5, 6). As shown in cultured fibroblasts blocked with ERA, ET-1 induction releases transforming growth factor–β (TGF-β), a profibrogenic protein and ERK 1/2, a proliferative agent (5, 6) which are important markers in vascular proliferation. Furthermore, ET-1 also enhances the over-expression of alpha-smooth muscle actin (α-SMA) and the deposition of collagen (5) leading to fibrosis. Again, in immune cells, the expression of ET_AR induces the release of interferon-gamma (IFN-γ), a proinflammatory mediator (55). Given the data on the pathogenicity of the endothelin system, several drug targets are recommended (56). However, the

propensity of other ligands, including AAbs, regulating ET_AR signaling warrants further investigations to understand the specific intracellular mechanisms involved.

1.4 Contribution of autoantibodies to SSc

The pathology of SSc is characterized by a defective immune system that leads to the AAbs production [1, 3]. It is therefore described that detectable AAbs present in the sera/plasma of SSc patients are involved in the pathogenesis [7, 57-59].

1.4.1 Autoantibodies targeting nuclei fragments and cytoplasmic proteins

Cytoplasmic and nuclear fragment AAbs were the first AAbs discovered in SSc patients (57). Anti-nuclear antibodies (ANA) are frequently detected in SSc patients and serve as valuable diagnostic biomarkers (57). For example, anti-topoisomerase I antibodies (ATA) are detectable in about 15 to 42% of SSc patients and are associated with a high risk of digital ulcers, pulmonary arterial hypertension (PAH), and cardiovascular complications (57). ATA are also associated with kidney damage and increased mortality (57). Furthermore, anti-centromere antibodies (ACA) are predominantly present in SSc cohorts, but these patients are at a lower risk for PAH and cardiovascular complications than ATA cohorts (57). Anti-ribonucleic acid polymerase antibodies (anti-RNAP) are associated with kidney complications (57). However, contrary to ATA and ACA, anti-RNAP are associated with a better prognosis (57). In addition, AAbs targeting non-nuclear targets such as EC, fibroblasts, and platelet-derived growth factors are emerging as essential contributors to SSc pathology (4, 57, 58). Interestingly, despite the high presence of cytoplasmic and nuclear AAbs in SSc patients, their functional role remains unclear (57). Few studies predict that AAbs induce endothelial dysfunction, cytotoxicity, apoptosis, and monocyte adhesion that facilitate SSc pathology (57, 58), but the motivating mechanisms are not fully elucidated.

1.4.2 Autoantibodies against GPCR in SSc

Detectable GPCR-AAbs in SSc patients are recognized in the modulation in the pathogenesis of SSc (58, 59). Accumulating evidence suggests GPCR-AAbs are potent modulators of immune and non-immune cell mechanisms that enhances the pathogenesis of SSc (7, 59, 60). Remarkably, AAbs against the vascular receptors, AT_1R and ET_AR (AT_1R-AAbs and ET_AR-AAbs) are described to be associated with SSc pathology (7, 58, 59).

1.4.2.1 Angiotensin II type 1-receptor autoantibodies in SSc

The AT_1R stimulated by angiotensin II is a key regulator of the vascular tone, the dysregulation of which leads to kidney and cardiovascular damage (61). Similarly, AT_1R-AAbs targeting the ECL-2 of AT_1R regulate AT_1R signaling, resulting in vascular crises in autoimmune and vascular diseases (8, 62). Mechanistically, SSc-AT_1R-AAbs are known to induce the release of proinflammatory mediators, including interleukin-8 (IL-8) and chemokine (C-C motif) ligand 18 (CCL18), and pro-fibrotic proteins collagen type I in both immune and non-immune cells (11, 12). In EC, SSc-AT_1R-AAbs activate ERK 1/2 and TGF-β (10) and also promote cell proliferation. Recently, AT_1R-AAbs from SSc patients were shown to induce cell proliferation via $G_{q/11}$-mediated NFAT activation (46). There is also evidence of an enhanced vasoconstrictive effect with additive stimulation of angiotensin and AT_1R-AAbs (14). Pre-sensitization of mice with AT_1R-AAbs also provides evidence for Ca^{2+} release and α-SMA expression, anticipating a role in SSc pathogenesis (15). A recent *in vivo* study in which mice were immunized with AT_1R-AAbs showed inflammation of the skin and lungs with reduced apoptotic activity indicating pathogenic effects of AT_1R-AAbs (63).

1.4.2.2 Endothelin type A receptor autoantibodies in SSc

The presence of ET_AR-AAbs in the sera/plasma of SSc patients is also predictive of pathology (7, 10). Data from *in vitro* studies show the agonistic effect SSc ET_AR-AAbs in endothelial cells to activating the release of pro-mitogenic ERK 1/2 and pro-fibrogenic TGF-β, key mediators in SSc pathogenesis (10). Similarly, ET_AR-AAbs also promote interleukin-8 (IL-8), vascular adhesion molecule-1 (VCAM-1) release and neutrophil migration in endothelial cell lines, all of which are also known mediators of SSc pathogenesis (11). Indeed, ET_AR-AAbs mobilize IL-8 and chemokine (C-C) ligand 8 (CCL8) release in immune cells, showing a role in immune cell activation (12). In addition, ET_AR-AAbs from SRC patients induced ERK 1/2 phosphorylation and Ets-1 regulation in endothelial cells (13). Passive transfer of ET_AR-AAbs into healthy C57BL/6J mice also revealed an increase in neutrophils in the bronchial lavage fluid, indicating inflammatory induction (11). Furthermore, perfusion of rat lungs with ET_AR-AAbs isolated from SSc-PAH patients showed an elevated Ca^{2+}, which was blocked by ERA, suggesting a stimulatory function via ET_AR (15). In the same study, passive transfer of ET_AR-AAbs showed increased expression of α-SMA and vasculopathy (15). The pathogenic value of ET_AR-AAbs, which require specific therapeutic targeting, is therefore promising.

2 Hypothesis and objectives

2.1 Hypothesis

The endothelin system is an important pharmacological target in vasculopathies including systemic sclerosis (SSc) (6, 16). In SSc in particular, endothelin-1 (ET-1)-induced activation of the endothelin type A receptor (ET_AR) shows pathological signatures of inflammation, fibrosis and proliferation that are difficult to treat (1, 5). Although endothelin receptor antagonists (ERAs) help to slow the progression of the SSc fibrotic picture (6), there is an insufficient understanding of the ligand-binding dynamics and functional selectivity. In the meantime, autoantibodies (AAbs) against ET_AR have also emerged as agonistic modulators of SSc pathology (7, 58). However, the ligand binding and functional selectivity is unknown. But the disclosed crystal structure of the endothelin type B receptor (ET_BR) which shares about 60% structural homology with ET_AR suggests the involvement of the extracellular amino terminus (Nter) domain in ligand binding and functionality (24, 25, 37). Moreover, previous mutagenesis and molecular docking studies have predicted the involvement of the ET_AR-Nter in ligand binding (21, 28, 64, 65). Given that the ET_AR-Nter is the most distinct domain of GPCRs and is readily accessible to ligands (AAbs), it is hypothesized that the relatively large molecular sized ET_AR-AAbs recognize different regions of the ET_AR-Nter than the natural ligand ET-1 to trigger specific signaling mechanisms.

2.2 Objectives

To validate the hypothesis that ET_AR-AAbs bind to ET_AR-Nter on different sites to trigger signaling mechanisms different from ET-1, mammalian cell-based systems were used. The objectives were as follows:

1. To determine which parts of ET_AR-Nter affect ET-1-mediated activation of G-proteins, $G_{q/11}$, $G_{12/13}$, and ERK 1/2 using luciferase reporter assays.
2. To determine whether ET_AR-AAbs bind to different parts of ET_AR-Nter than ET-1 and initiate specific activation of $G_{q/11}$, $G_{12/13}$ and ERK 1/2 using luciferase reporter assays.
3. To determine if ET_AR-AAbs recognize different parts of ET_AR-Nter and induce cAMP production other than ET-1 using a cAMP ELISA assay.

3 Materials and Methods

3.1 Materials

Reagents, Solutions	Company
10X MuLV Reverse Transcriptase Buffer	New England BioLabs
10X Trypsin-EDTA	PAA
3.7 % Formaldehyde	Charite Management Facility
5X PLB	Promega
Agarose	Serva
Ampicillin	Alkom
Bacto agar	Becton Dickinson (BD) Biosciences
Bacto peptone	BD Biosciences
Bacto tryptone	BD Biosciences
Bacto yeast extract	BD Biosciences
DMSO	Sigma Aldrich
dNTP mix	Thermo Fisher Scientific
DMEM	BioWest
1X Dulbecco's PBS	Gibco
Ethanol 99.8%	Carl Roth
FCS	Gibco
Lipofectamine™ 3000	Thermo Fisher Scientific
Midori Green Advance DNA Stain	NIPPON Genetics
NaCl powder	Lonza
Penicillin/Streptomycin	PAA
Poly-L-lysine	Sigma Aldrich
Q5 DNA Polymerase	New England BioLabs
RNase inhibitor	Thermo Fisher Scientific
MuLV reverse transcriptase	Thermo Fisher Scientific
Triton®X-100	Sigma Aldrich
Trypan blue	Sigma Aldrich
SOC	Carl Roth

Equipment

Equipment	Company
Biofuge primo R Centrifuge	Thermo Fisher Scientific
FLUOstar OPTIMA Microplate Reader	BMG LABTECH
Heidolph Titramax 100	Heidolph, Germany
Hera cell 240 Incubator	Thermo Electron Corporation
HERA safe Microbiological Safety Cabinet	Thermo Electron Corporation
HiTrap Protein G HP	GE Healthcare
Incubating Orbital Shaker professional 3500	VWR
Multifuge 1s-R Centrifuge	Thermo Electron Corporation

Neubauer Counting Chamber	Carl Roth
ND-1000 Spectrophotometer	VWR
Polymax 1040 T Platform Shaker	Heidolph
T Professional Thermocycler	Biometra
UV-transilluminator Gene Flash	Syngene

Kits

Kits	**Company**
cAMP ELISA kit	Enzo life sciences
GeneJET Plasmid Miniprep	ThermoFisher Scientific
Luciferase Assay System	Promega
Nano-Glo® HiBiT Extracellular Detection System	Promega
NucleoBond Xtra Midi	Macherey-Nagel
Q5® Site Directed Mutagenesis	New England BioLabs Inc.

Plasmid, bacteria, cell line, and enzymes

Plasmid	**Company**
pcDNA 3.1	ThermoFisher Scientific

Bacteria	**Company**
NEB 5-alpha Competent *E.coli* Cells	New England Biolabs

Cell line	**Company**
Human embryonic kidney 293 T cell line (HEK 293 T)	ATCC

Enzyme	**Company**
*Hind*III-HF	New England Biolabs

Agonist

Agonist	**Company**
Endothelin-1 human and porcine	Sigma Aldrich
Bovine thyroid-stimulating hormone	Sigma Aldrich

Buffer Recipes

Buffer	**Reagent**	**Final Conc.**
Binding buffer, pH 7.0	Na_2HPO_4 In ddH_2O Filtered (0.45 µm)	20 mM
Elution buffer, pH 2.7	Glycin-HCl In ddH_2O Filtered (0.45 µm)	0.1 M

Neutralization buffer, pH 9.0	Tris-HCl In ddH_2O Filtered (0.45 µm)	1 M
TBE buffer 1X, pH 8.0	Tris Boric acid EDTA In ddH_2O	89 mM 89 mM 2 mM
DNA sample loading buffer 6x	Glycerol Xylene cyanide Bromophenol blue In ddH_2O	30% v/v 0.25% v/v 0.25% m/v
PBS (Ca^{2+}- /Mg^{2+} -free), pH 7.3	NaCl KCl Na_2HPO_4 KH_2PO_4 In ddH_2O (*Sterilized by autoclaving*)	137 mM 2.7 mM 9 mM 2.3 mM
Ampicillin stock solution	Ampicillin powder In ddH_2O	100 mg/mL

Bacteria culture media

Lysogeny broth (LB)	**Conc.**
Bacto tryptone	1% m/v
Bacto yeast extract	0.5% m/v
NaCl	1% m/v
	add ddH_2O

Sterilized by autoclaving

LB Agar	**Conc.**
Bacto tryptone	1% m/v
Bacto yeast extract	0.5% m/v
NaCl	1% m/v
Bacto agar	1.5% m/v
	add ddH_2O

Sterilized by autoclaving

LB with Ampicillin	**Conc.**
Ampicillin stock solution	100mg/mL
Bacto tryptone	1% m/v
Bacto yeast extract	0.5% m/v
NaCl	1% m/v
	add ddH_2O

Sterilized by autoclaving

LB Agar with Ampicillin	Conc.
Sterilized LB Agar	
Ampicillin stock solution	100mg/mL

LB broth with Ampicillin	Conc.
Sterilized LB broth	
Ampicillin stock solution	100mg/mL

Cell culture media

Complete medium

Components	Final Concentration
DMEM High Glucose	500mL
Penicillin	10000 units/mL
Streptomycin	10mg/mL
L-Glutamine	2mM
Fetal Calf Serum	10% v/v
Sodium Pyruvate	1mM
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	10mM, pH 7.3

Starvation Medium

Components	Conc
DMEM High Glucose	500mL
Penicillin	10000 units/mL
Streptomycin	10mg/mL
L-Glutamine	2mM
Sodium Pyruvate	1mM
HEPES	10mM, pH 7.3

Primers

Three mutagenic constructs were generated to test the involvement of ET_AR-Nter in ligand binding. Primers were designed using the online software NEBaseChangerTM (https://nebasechanger.neb.com, 10/03/2020). Biolegio (Netherlands) supplied site-directed mutagenesis primers listed in Table 1. The primers listed in Table 2 were used in the sequence evaluation of the constructs.

Table 1: Primers for mutagenesis

Constructs name	**5'- to -3'**
ET_AR-HiBiT-Nter del AA 46-65	F: CACAACTATTGCCCACAG R: GAGCTCTGTGCCACGAAA
ET_AR-HiBiT-Nter del AA 26-65	F: CACAACTATTGCCCACAG R: TCTCTCAGGATTATCACTG
ET_AR-HiBiT-Nter del AA 2-65	F: CACAACTATTGCCCACAG R: ACCTGAACTTCCGCTAATC

Table 2: Primers for sequencing

Sequencing primers	**5'- to -3'**
CMV (pcDNA 3.1 plasmid, nucleotides 769 to 789)	F: CGCAAATGGGCGGTAGGCGTG
pcDNA 3.1 del AA 46-65	F: CACAACTATTGCCCACAG
pcDNA 3.1 del AA 26-45	F: TCTCTCAGGATTATCACTG
pcDNA 3.1 del AA 2-25	F: ACCTGAACTTCCGCTAATC

3.2 Methods

3.2.1 Generation of ET_AR N-terminus constructs

To generate the ET_AR-Nter mutagenic constructs, the human complementary deoxyribonucleic acid (cDNA) from *EDNRA*, coding for ET_AR, tagged with a short peptide sequence called HiBiT (11 AA) that had been previously generated in the laboratory was used. The HiBiT tag served to assess ET_AR expression as described (66). The resulting construct ET_AR-HiBiT-WT, cloned into pcDNA 3.1, was subsequently used as a template to generate the mutagenic constructs.

3.2.1.1 Site-directed mutagenesis

Three deletions of ET_AR-Nter were generated sequentially. Initially, ET_AR-HiBiT-WT was amplified to delete AA 46-65 of ET_AR-Nter to obtain the construct ET_AR-HiBiT-Nter del AA 46-65 using the Q5® site-directed mutagenesis kit. Later, ET_AR-HiBiT-Nter del AA 46-65 was used as a template to delete additionally AA 26-45 to generate the ET_AR-HiBiT-Nter del AA 26-65 construct. Finally, ET_AR-Nter AA 2-25 were further deleted using the ET_AR-HiBiT-Nter del AA 26-65 as a template to generate ET_AR-HiBiT-Nter del AA 2-65. All amplification experiments were performed using the reaction components provided in the Q5® site-directed mutagenesis kit and under specific polymerase chain reaction conditions supplied by the NEBaseChanger™

software and listed in Tables 3 and 4.

Table 3: Reaction components for exponential amplification of ET_AR-HiBiT-Nter constructs

Components	**Volume**	**Final Concentration**
Q5 Hot Start High-Fidelity 2X Master Mix	12.5µL	1X
10 µM forward Primer	1.25µL	0.5µM
10 µM reverse Primer	1.25µL	0.5µM
Template DNA (1-25 ng/ µl)	1µL	10ng
Nuclease-free water/ddH_2O	9.0µL	

Table 4: Conditions of PCR for the mutagenic constructs

Constructs	**Second denaturation (temp and time)**	**Annealing (temp and time)**	**Elongation step (temp and time)**
ET_AR-HiBiT-Nter del AA 46-65	98°C 10 s	62°C 30 s	72°C 2 min
ET_AR-HiBiT-Nter del AA 26-65	98°C 10 s	58°C 30 s	72°C 2 min
ET_AR-HiBiT-Nter del AA 2-65	98°C 10 s	62°C 30 s	72°C 2 min

After exponential amplification and construction of mutagenic constructs, the PCR fragments were repaired and ligated with the enzyme Kinase-Ligase-*DpnI* (KLD) according to the Q5® protocol for site-directed mutagenesis kit. The KLD treatment protocol is listed in Table 5 below.

Table 5: Components for KLD treatment

Components	**Volume**	**Final Conc.**
PCR Product	1µL	
2X KLD Reaction Buffer	5µL	1X
10X KLD Enzyme Mix	1µL	1X
Nuclease-free Water	3µL	

To amplify the ET_AR-HiBiT-Nter plasmids, 5µL of the KLD reaction product were transformed into 50µL of thawed NEB 5-alpha *E. coli* competent cells, whose genotype is shown in Table 6.

3.2.1.2 Bacterial Transformation

To amplify the ET_AR-HiBiT-Nter plasmids, 5µL of the KLD reaction product were transformed into 50µL of thawed NEB 5-alpha *E. coli* competent cells, whose genotype is shown in Table 6.

Table 6: Bacteria genotype

Bacteria	**Provider**	**Genotype**
NEB 5-alpha Competent *E.coli*	New England Biolabs	*fhuA2, Δ(argF-lacZ) U169, phoA, glnV44, Φ80Δ(lacZ)M15, gyrA96, recA1, relA1, endA1, thi-1, hsdR17*

The transformed bacteria were first incubated on ice for 30 min. The mixture was then heat shocked at 42°C for 30 s to create pores for entry of deoxyribonucleic acid (DNA) and incubated again on ice for 5 min. The bacteria were later allowed to recover by gently shaking the competent cell mixture in 950 µL of superoptimal catabolite broth (SOC) for 1 hour at 37 °C. Then, 100- and 1000-fold dilutions were made in SOC and seeded to obtain bacterial colonies on Lysogeny Broth (LB) plates containing ampicillin (LB Amp). The plates were subsequently incubated at 37°C overnight.

3.2.1.3 Mini-prep

After overnight incubation, the Gene JET Miniprep kit was used to isolate the plasmid constructs from the transformed bacteria. Here, single pure colonies that had grown on the LB-Amp plates were isolated. They were individually inoculated into 3 mL LB-Amp. The 3 mL mixtures containing bacterial colonies were then incubated overnight at 37°C with shaking at 180 revolutions per minute (rpm). Later, 2 mL of the overnight culture was pipetted into Eppendorf tubes, centrifuged, resuspended, lysed, neutralized and eluted according to the Gene JET Miniprep Kit protocol to isolate the plasmids. The eluted plasmid concentration was measured with a Nanodrop® spectrophotometer, and the plasmids were stored at -20°C.

3.2.1.4 Screening for positive plasmids

The isolated coiled plasmids were digested with a restriction enzyme, *Hind*III, to linearize them. Then, 14µL of the digested plasmids and a 1kbp ladder were loaded on an agarose gel with 1X loading buffer and 0.05X Midori green to screen for the positive plasmids. The samples were run by electrophoresis at 100 volts for 1 hr. The gel was visualized under a SysGene Ultraviolet illuminator. Bands at a size of 6.2kbp corresponding to the plasmid plus insert were considered positive.

3.2.1.5 Sequencing and alignment

For the specific evaluation of the deleted sequence constructs, electrophoresed positive plasmids were combined with sequence primers listed in Table 2 and sequenced commercially by LGC Genomics GmbH. The sequencing reports from the company were analyzed using the DNABaser sequence alignment software (Heracles' BioSoft SRL Romania).

3.2.1.6 Midi-prep

To obtain a high plasmid yield for cell culture experiments, selected plasmids were further isolated using the Nucleobond® Xtra Midiprep kit according to the manufacturer's instructions. Single pure colonies were picked from the LB-Amp plate and incubated in 3mL LB-Amp. The mixture was then incubated at 37°C for 8 hrs with shaking. The pre-culture was diluted at 1:500 for deleted constructs and 1:1000 for the WT receptor in LB-Amp in 100mL final volume. The dilutions were incubated at 37°C overnight with shaking at 180rpm. The overnight culture was later lysed, neutralized, precipitated and eluted according to the manufacturer's instructions. The eluted plasmid's concentration was measured with a Nanodrop® spectrophotometer. The plasmids with concentrations above 1000ng/mL were stored at -20°C for further experiments.

3.2.1.7 Glycerol Stock

To prevent plasmid degradation, the bacteria containing the chosen plasmids were mixed with glycerol. Here, single colonies were picked and inoculated into LB Amp broth and shaken at 37°C for eight hrs. Then 250µL of the pre-culture were added to 750µL of 60% glycerol, mixed and frozen at -80°C.

3.2.2 Cell culture handling and transfection

3.2.2.1 Cells culture conditions

The human embryonic kidney 293 T cells (HEK 293 T) were maintained in Dulbecco's modified Eagle medium (DMEM) High Glucose supplemented with 10% fetal calf serum (FCS), penicillin/streptomycin, and sodium pyruvate and buffered in HEPES (complete medium). The HEK 293 T were incubated in a humidified atmosphere with 5 % CO_2 at 37°C. Nitrogen-frozen cells were thawed, plated in T75 flasks, and cultured for five days to reach 80% confluence. Confluent cells were washed in 1X phosphate buffered saline (PBS) and detached in 1X trypsin for 3 min. Detached cells were resuspended in complete medium and centrifuged at 3000rpm for 3 min at room temperature. The supernatant of the centrifuged cells was removed, and the cell

pellet was resuspended in complete medium. 20µL of cell suspension was stained with Trypan blue, charged on a Neubauer counting chamber and counted under a microscope. Depending on the nature of the experiments, optimized cell numbers were selected and seeded into 12-, 24- or 96-well plates precoated with Poly-L-lysine. Moreover, 2 million cells were seeded into a T75 flask to keep the cells in culture.

3.2.2.2 Cell transfection

Cells at 80% confluence were transfected with 50ng/µL, 200ng/µL and 400ng/µL plasmid in 96-, 24- or 12-well plates, respectively, with Lipofectamine 3000^{TM} according to the manufacturer's protocol. Transfection was performed in starvation medium for 4hrs. After this time, starvation medium was replaced with complete medium.

3.2.3 Patient samples and cell stimulation

3.2.3.1 Patients characteristics and ethical approval

SRC patients who underwent treatment with angiotensin converting enzyme inhibitors (ACEI) between 2006 and 2010 at the Department of Nephrology and Medical Intensive Care at Charité – Universitätsmedizin Berlin and were refractory to the drug were recruited. The study was submitted to the local ethical committee (EA1/013/705) and patient informed consent was obtained. SRC was defined clinically as an unexplained decrease in renal function following a ≥ 50% increase in serum creatinine and biopsy evidence of obliterative vasculopathy in SSc patients.

3.2.3.2 Isolation of patients' immunoglobulin G

Plasma samples of healthy and SRC samples were collected and the whole IgG was isolated using the using HiTrap® Protein G high performance (HP) columns. The collected plasma samples were first filtered through a 0.45µm filter to remove debris and then diluted 1:2 with binding buffer before running through the HiTrap® Protein G HP column twice. The column was rinsed with binding buffer to eliminate non-specific binding. The IgG solution was then eluted and neutralized. IgG was finally dialyzed against DMEM Low Glucose overnight. The concentration of the IgG dialyzed preparation was measured by Labor Berlin GmbH. IgGs were stored at -80°C and thawed once for experiments. Subsequently, IgG dialyzed preparations were tested for the presence of ET_AR-AAbs using sandwich ELISA (Cell Trend GmbH) as previously described (14). Cells were stimulated with positive IgG preparations (ET_AR-AAbs level above 17 U/mL) and the measured IgG concentration of these preparations was used to calculate the stimulation concentration of 1.0mg/mL.

3.2.4 Stimulation of cells

Transfected cells were starved overnight prior to stimulation. Then, cells were stimulated with different concentrations of ET-1, 10^{-7}M, 10^{-8}M and 10^{-9}M, and patient ET_AR-AAbs (1.0mg/mL) or with DMEM Low Glucose as non-stimulated control. Stimulated cells were incubated for 6hrs for luciferase reporter assays and 45mins for cAMP assay with non-stimulated and stimulated controls, respectively.

3.2.5 Nano-Glo® HiBiT extracellular detection system

The Nano-Glo® HiBiT extracellular detection system utilizes the principle that an 11-amino acid peptide called HiBiT tagged to a receptor will luminesce when coupled to a large protein of about 17.6 kDa called LgBiT tagged with a NanoLuc® luciferase after a color developer fumarizine is added (46, 67). The measured luminescence signal is proportional to the number of labelled receptors on the cell surface. In the assay, 20,000 HEK 293 T were seeded into white 96-well Corning plates. The cells were later transfected with 50ng/µL plasmid after four days of incubation. On the day of the experiment, the cell culture medium was removed and replaced with 100µL of a mixture containing Nano-Glo® HiBiT extracellular buffer 1X, extracellular substrate 2% v/v, and LgBiT protein 1% v/v according to the kit's alternative protocol. The plates were then sealed in an aluminium foil to prevent light exposure and gently shaken for 15 mins. The relative luminescence of the assay was measured using the FLUOstar OPTIMA Microplate Reader.

3.2.6 Luciferase reporter assay

The luciferase reporter assay system used in this work is an indirect method to assess G-proteins activation (45, 46, 68). In these assays, the binding sites of the relevant transcription factors were added in the promoter region of the *Renilla* luciferase (46, 68). During G-protein activation, transcription factors are in-turn activated and bind to their respective promoter sites located in the *Renilla* luciferase triggering luciferase production measured by luminescence. In the experiments, the transcription factors, NFAT, SRF and SRE monitoring the activation of $G_{q/11}$, $G_{12/13}$ and ERK 1/2, respectively were used (45, 68, 69). In each reporter assay, 100,000 seeded cells into 24-well plates were transfected with 200ng/µL of WT or mutagenic plasmid and reporter plasmid. Starved cells were stimulated for six hrs, washed with 1X PBS and lysed with 1X phosphate lysis buffer (PLB) under shaking for 15 min. Then 60 µL of lysate was added to 40 µL to fumarizine luciferase color developer. Relative luminescence signals were measured using the FLUOstar OPTIMA Microplate Reader.

3.2.7 cAMP ELISA assay

The cAMP ELISA kit was used to determine ligand-induced cAMP production. In this experiment, 12-well plates were seeded with 250,000 cells per well. Cells were transiently transfected with 400ng/µL WT or mutant constructs. After overnight starvation of the transfected cells, half of the starvation medium was removed and replaced with 100µg/mL 3-isobutyl-1-methylxanthine (IBMX) to block the activity of phosphodiesterase (PDE) for one hr. Then, cells were stimulated with ET_AR-AAbs or ET-1 for 45 min. Stimulated cells were detached with 0.1M hydrochloric acid (HCl) for 15 min under gentle shaking. Detached cells were centrifuged for 3 min at 3000rpm at 4°C. Lysates were then processed according to the ELISA kit manufacturer's protocol. The absorbance of the produced cAMP was measured at a wavelength of 405 nm using the FLUOstar OPTIMA Microplate Reader. The results were analyzed using the 4-parameters logistic regression with standards supplied by the manufacturer.

3.2.8 Statistical analysis

Data from repeated experiments were analyzed using the Graph Pad Prism 9.0 software. Data are represented as mean ± standard error of mean (SEM), and n represents the number of independent experiments. Kruskal-Wallis test was used to compare the differences between groups and post-hoc tests were further explored. $p< 0.05$ was considered statistically significant.

4 Results

ET-1 and ET_AR-AAbs are critical players in the pathophysiology of SSc (5, 58). However, the binding domain(s) to their cognate receptor, ET_AR, are inadequately described. Intracellular signaling events induced by ET-1- and ET_AR-AAbs are also unknown. Since the extracellular ET_AR-Nter is most accessible to ET-1 and ET_AR-AAbs, this work investigated whether the two ligands recognize different parts of the ET_AR-Nter to initiate specific signaling mechanisms. For this work, constructs bearing sequential deletions of ET_AR-Nter, ET_AR-HiBiT-Nter del AA 46-65 (AA 46-65 were deleted), ET_AR-HiBiT-Nter del AA 26-65 (AA 26-45 were deleted in addition to 46-65) and ET_AR-HiBiT-Nter del AA 2-65 (AA 2-25 were deleted in addition to 26-65) were expressed in HEK 293 T and activation of G-proteins, and ERK 1/2, as well as production of cAMP, were examined. In each case, plasmids triggering the over expression of WT or deleted human ET_AR were transiently transfected.

4.1 Loss of ET_AR-Nter AA 2-25 promotes membrane expression

The Nter of GPCRs may play a role in cell trafficking and membrane expression (70). However, the role of ET_AR-Nter in membrane expression has not been studied. Therefore, the plasma membrane expression of deleted ET_AR-Nter constructs was assessed using the Nano-Glo® HiBiT assay. As shown in figure 4, in transiently transfected cells, the relative membrane expression of ET_AR-HiBiT-Nter del AA 2-65 was significantly higher than that of ET_AR-HiBiT-WT. In contrast, there was no difference in the relative expression of ET_AR-HiBiT-Nter del AA 46-65 and ET_AR-HiBiT-Nter del AA 26-65 compared to ET_AR-HiBiT-WT.

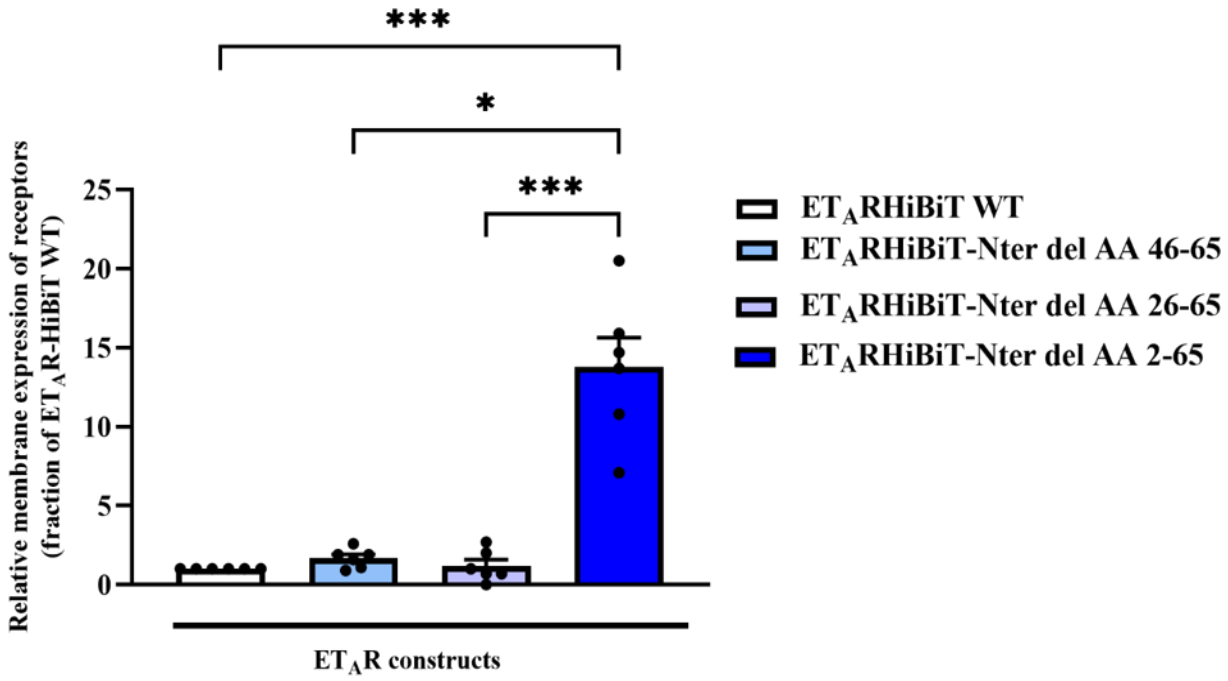

Figure 4: Deletion of ET$_A$R-Nter AA 2-25 enhances relative membrane expression of ET$_A$R.
ET$_A$R expression on the cell surface was assessed in HEK 293 T transfected with ET$_A$R-HiBiT-WT and deleted constructs; ET$_A$R-HiBiT-Nter del AA 46-65, ET$_A$R-HiBiT-Nter del AA 26-65 and ET$_A$R-HiBiT-Nter del AA 2-65. Data are shown as mean ± SEM of six independent experiments, * $p<0.05$, *** $p<0.001$ (Post-hoc tests after performing a Kruskal-Wallis test).

The data show that deletion of AA 2-25 from ET$_A$R-Nter promotes the relative plasma membrane expression of ET$_A$R.

4.2 ET$_A$R-Nter triggers dose-dependent ET-1-mediated activation of G-protein and ERK 1/2

The contribution of ET$_A$R-Nter to ET-1 binding and signaling is imprecise. Therefore, the influence of ET$_A$R-Nter domains on ET-1-mediated G-protein and ERK 1/2 activation was examined. NFAT, SRF and SRE-activated reporter plasmids monitoring activation of $G_{q/11}$, $G_{12/13}$ and ERK 1/2 respectively, were co-transfected into HEK 293 T cells with ET$_A$R-Nter deleted constructs. Luminescent activity of dose-dependent ET-1 stimulation of reporter plasmids was quantified.

4.2.1 Different ET_AR-Nter domains trigger ET-1-induced G-protein activation dose-dependently

Modelling and mutagenesis data suggest that ET_AR-Nter acts as a closed lid for ET-1 binding to ET_AR, thereby ensuring compactness and efficient signaling (23, 28). The study examined whether deletion of different ET_AR-Nter domains affects ET-1-mediated activation of $G_{q/11}$ and $G_{12/13.}$ As shown in the left part of the panels of figure 5A-C, ET-1 dose-dependently increased NFAT activation and thus $G_{q/11}$ activation in the ET_AR-HiBiT-WT construct compared to the non-stimulated control. There was no significant effect on ET-1-mediated activation of $G_{q/11}$ in the deleted constructs, ET_AR-HiBiT-Nter del AA 46-65 and ET_AR-HiBiT-Nter del AA 26-65 compared to ET_AR-HiBiT-WT construct as seen in figures 5A and 5B. In contrast, there was a significant decrease in ET-1-mediated activation of $G_{q/11}$ via ET_AR-Nter del AA 2-65 compared to ET_AR-HiBiT-WT, as observed in figure 5C.

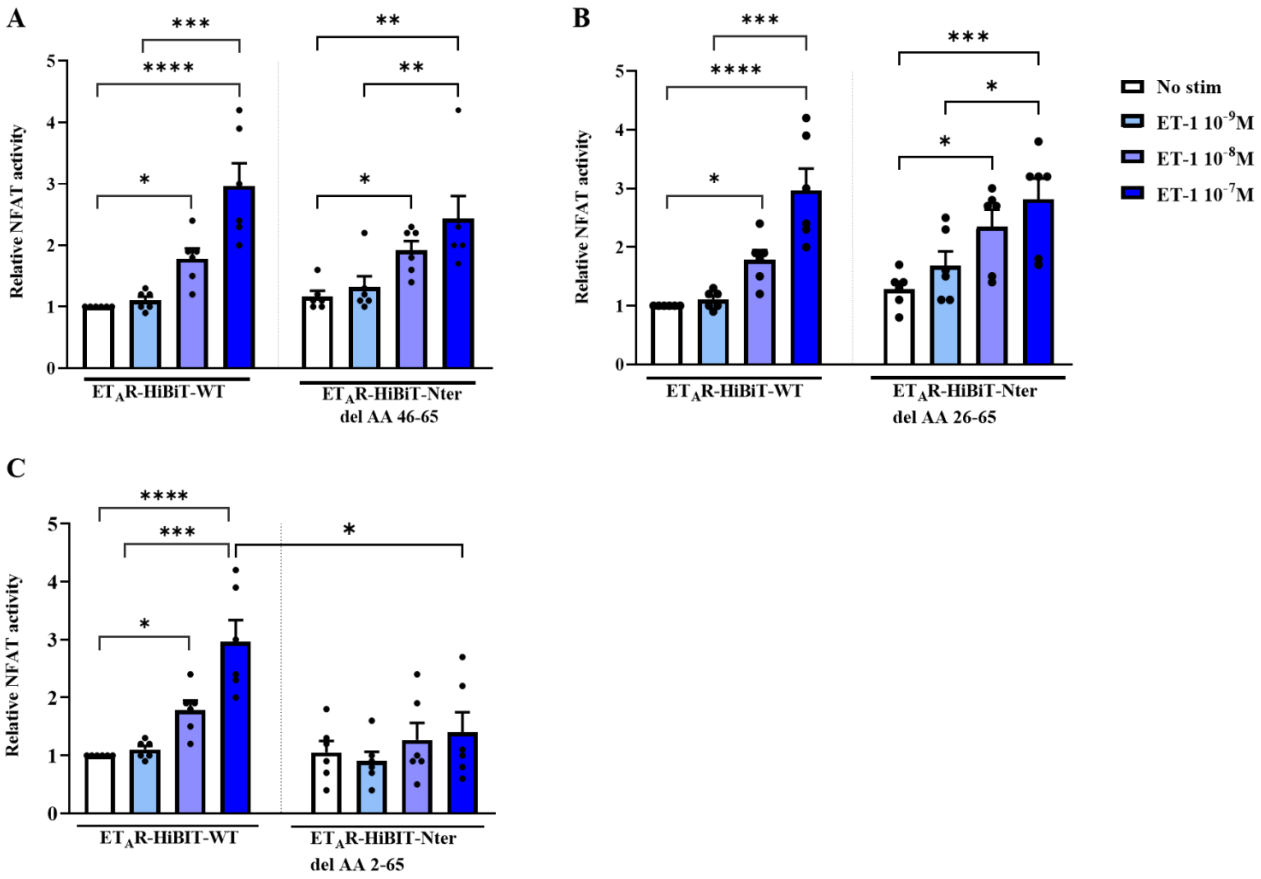

Figure 5: Deletion of ET_AR-Nter AA 2-25 decreases dose-dependent ET-1 activation of $G_{q/11}$.
HEK 293 T were transfected with ET_AR-HiBiT-WT or with **(A)** ET_AR-HiBiT-Nter del AA 46 to 65 **(B)** ET_AR-HiBiT-Nter del AA 26-65 and **(C)** ET_AR-HiBiT-Nter del AA 2-65. Transfected cells were treated with different doses of ET-1: 10^{-9}M, 10^{-8}M and 10^{-7}M. $G_{q/11}$ activation was monitored by NFAT-dependent luciferase production. Data are expressed as mean ± SEM of six independent experiments, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 (Post-hoc tests after performing a Kruskal-Wallis test).

The results show that while ET-1 dose-dependently increases $G_{q/11}$ activation but loss of ET_AR-Nter AA 2-25 abolishes this activation.

Furthermore, the effect of ET_AR-Nter on $G_{12/13}$ as reflected in SRF activation was monitored. As can be seen in the left part of the panels of figures 6A-C, ET-1 elicited a significant dose-dependent increase of $G_{12/13}$ activation in the ET_AR-HiBiT-WT compared to the non-stimulated control. Subsequent deletion of AA 46-65 produced no significant effect on ET-1-mediated activation of $G_{12/13}$ (figure 6A). However, further deletion of AA 26-45 resulted in a significant ET-1 dose-dependent increase in $G_{12/13}$ activation, as seen in figure 6B. Further deletion of AA 2-25 significantly decreased $G_{12/13}$ activation (figure 6C).

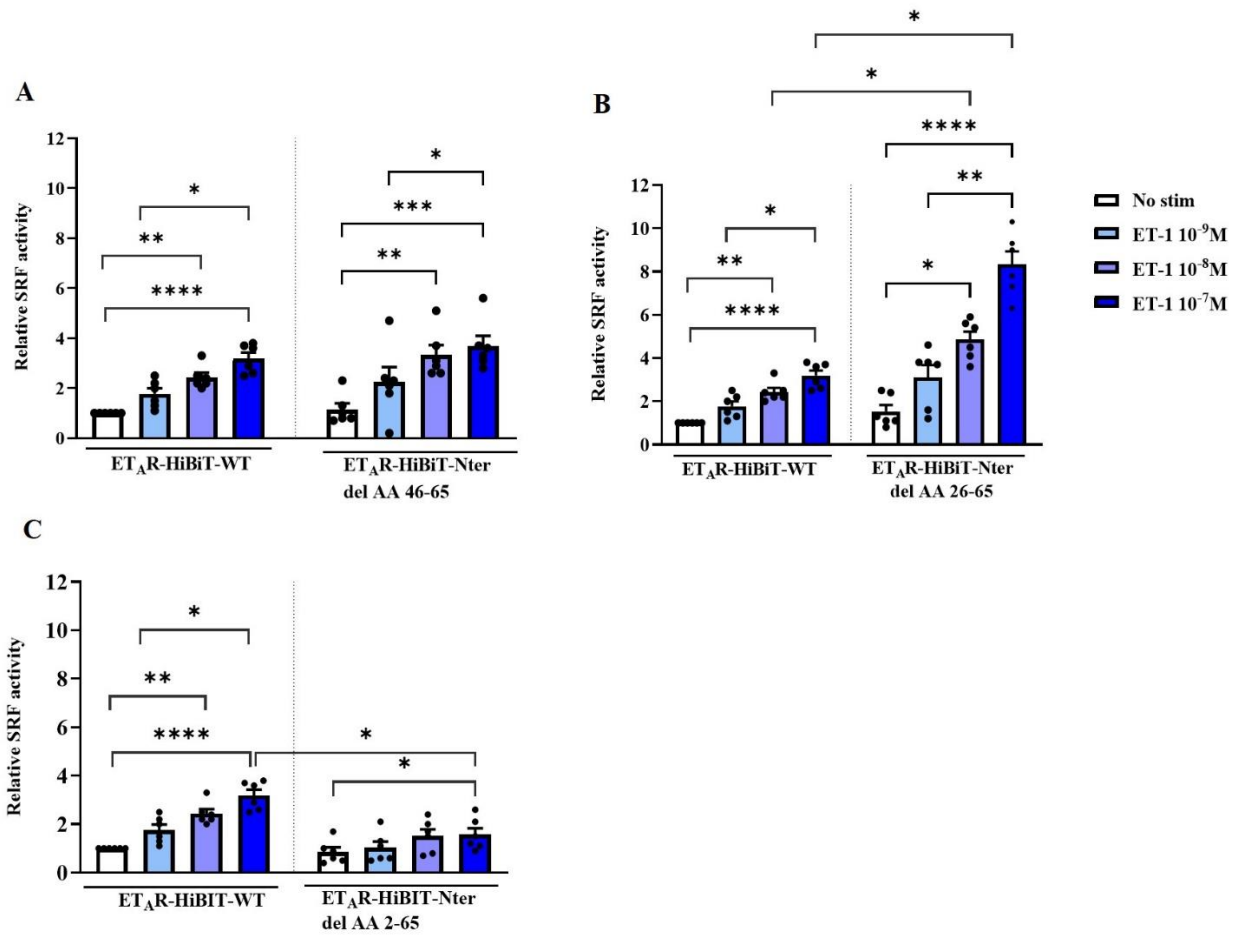

Figure 6: Deletion of ET$_A$R-Nter AA 26-45 and AA 2-25 affects dose-dependent ET-1-mediated activation of $G_{12/13}$.
HEK 293 T were transfected with ET$_A$R-HiBiT-WT or with **(A)** ET$_A$R -HiBiT-Nter del AA 46-65 **(B)** ET$_A$R -HiBiT-Nter del AA 26-65 and **(C)** ET$_A$R-HiBiT-Nter del AA 2-65. Transfected cells were stimulated with different doses of ET-1; 10^{-9}M, 10^{-8}M and 10^{-7}M. SRF-dependent luciferase luminescence was used to monitor $G_{12/13}$ activity. Data are expressed as mean ± SEM of six independent experiments, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ (Post-hoc tests after performing a Kruskal-Wallis test).

The data indicate that different AA domains of ET$_A$R-Nter are involved in the dose-dependent ET-1 activation of $G_{12/13}$. While AA 26-45 promote ET-1-induced $G_{12/13}$ activation, deletion of AA 2-25 abrogates activation.

Taken together, the data show that different parts of ET$_A$R-Nter participate in ET-1 binding to ET$_A$R to activate $G_{q/11}$ and $G_{12/13}$. AA 2-25 are required in ET$_A$R coupling to $G_{q/11}$ and $G_{12/13}$, but deletion of AA 26-45 increases $G_{12/13}$ activation. Of note, the deletion of AA 2-25 also caused increased

membrane expression of ET_AR, suggesting that AA 2-25 may be involved in ET_AR- trafficking and ET-1 binding to ET_AR.

4.2.2 Deletion of AA 2-25 abrogates ET-1-mediated activation of ERK 1/2

ET-1-induced activation of ERK 1/2 is indicated in the proliferative feature of SSc (5, 6). Therefore, it was further assessed if ERK 1/2 is activated in response to ET-1 binding to ET_AR and whether ET_AR-Nter is involved. The SRE reporter plasmid was used to assess the effect of ET_AR-Nter on ET-1-induced ERK 1/2 activation. As shown in the left part of figure 7, ET-1 significantly and dose-dependently increases ERK 1/2 activation in ET_AR-HiBiT-WT compared to the non-stimulated control. The deletion of AA 46-65 and AA 26-45 produced no effect on ERK 1/2 activation, as shown in the right parts of figures 7A and 7B. In contrast, the stimulatory effect was abolished upon deletion of AA 2-25, as shown in the right part of figure 7C. Nevertheless, a significant activating effect was observed for the highest ET-1 dose.

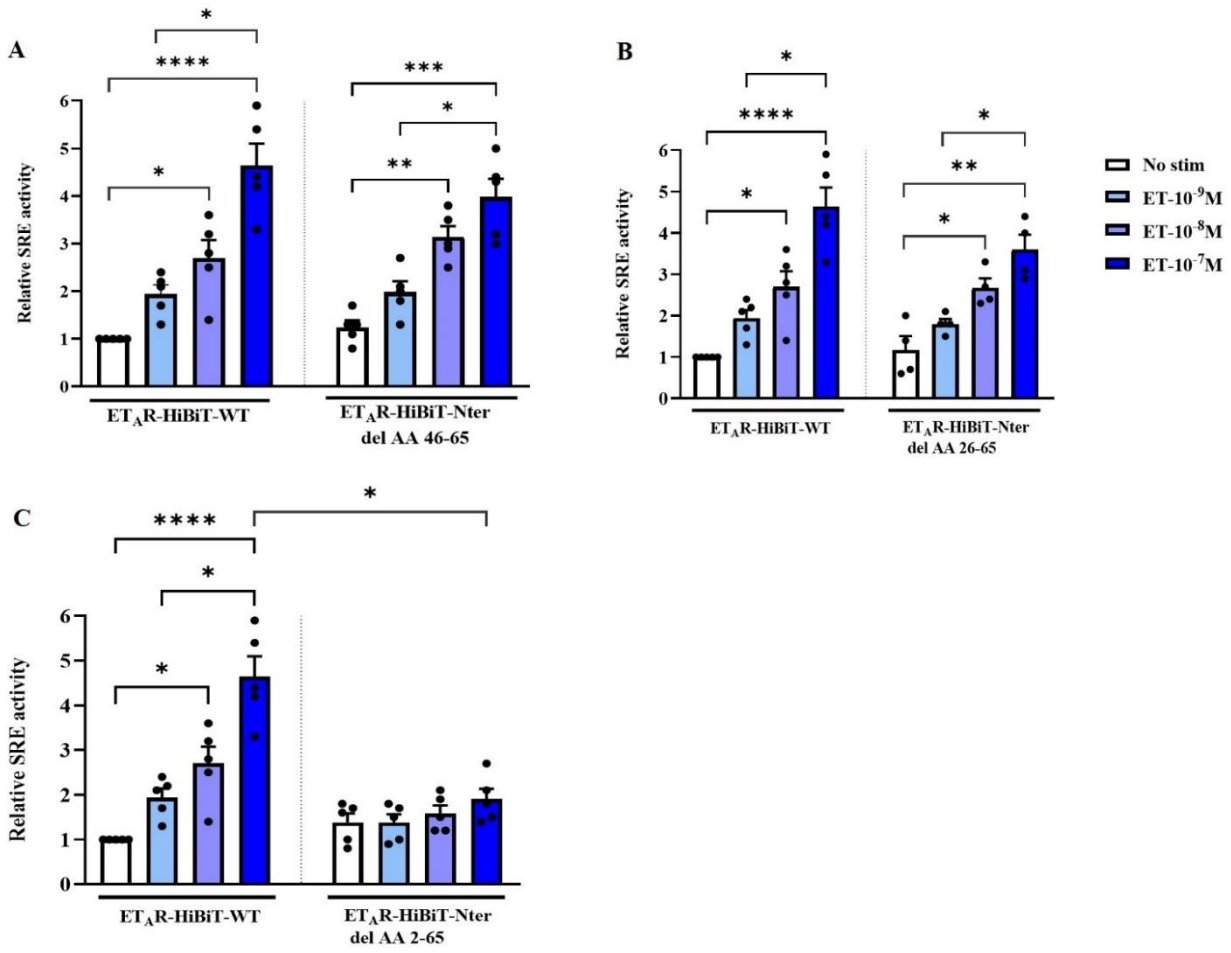

Figure 7: Loss of AA 2-25 abrogates ET-1-mediated activation of ERK 1/2.
HEK 293 T were transfected with ET_AR-HiBiT-WT receptor or with **(A)** ET_AR-HiBiT-Nter del AA 46-65 **(B)** ET_AR-HiBiT-Nter del AA 26-65 **(C)** ET_AR-HiBiT-Nter del AA 2-65. Cells were stimulated with 10^{-9}M, 10^{-8}M and 10^{-7}M of ET-1. ERK 1/2 activation was monitored with SRE-dependent luciferase luminescence. Data are expressed as mean ± SEM of five independent experiments, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ (Post-hoc tests after performing a Kruskal-Wallis test).

The data show that AA 2-25 is required for full activation of ERK 1/2. Since deletion of AA 2-25 also abolished $G_{q/11}$ activation, it can be extrapolated that $G_{q/11}$ activation is involved in ERK 1/2 activation (38).

In conclusion, it is observed that the intact ET_AR is required for dose-dependent ET-1 activation of G-proteins and ERK 1/2. However, deletion of different domains of ET_AR-Nter affects ET-1 binding to ET_AR and signaling. In particular, the AA 2-25 of ET_AR-Nter are required for ET-1-induced activation of ET_AR signaling.

4.3 ET_AR-Nter is involved in ET_AR-AAbs-mediated activation of G-protein and ERK 1/2

ET_AR-AAbs are emerging as effective modulators of ET_AR functionality in SSc (71). However, the ET_AR-AAbs binding domain(s) on ET_AR are unknown. Therefore, the influence of different domains of ET_AR-Nter on ET_AR-AAbs binding to ET_AR and signaling was investigated. Deleted ET_AR-Nter constructs were stimulated with 1mg/mL of ET_AR-AAbs and ET-1 10^{-8}M for comparison. The stimulatory effect on $G_{q/11}$, $G_{12/13}$ and ERK 1/2 activation was assessed by luciferase reporter assays.

4.3.1 Different ET_AR-Nter domains elicit ET_AR-AAbs-mediated activation of G-proteins

As shown in the left panels of figures 8A-C, ET_AR-AAbs stimulated $G_{q/11}$ activation significantly compared to the non-stimulated control. However, the stimulatory effect of ET-1 10^{-8}M was significantly stronger than that of ET_AR-AAbs. In contrast, deletion of ET_AR-Nter 2-25 produced a significant effect on the ET_AR-AAbs-induced activation of $G_{q/11,}$ as shown in figure 8A-C.

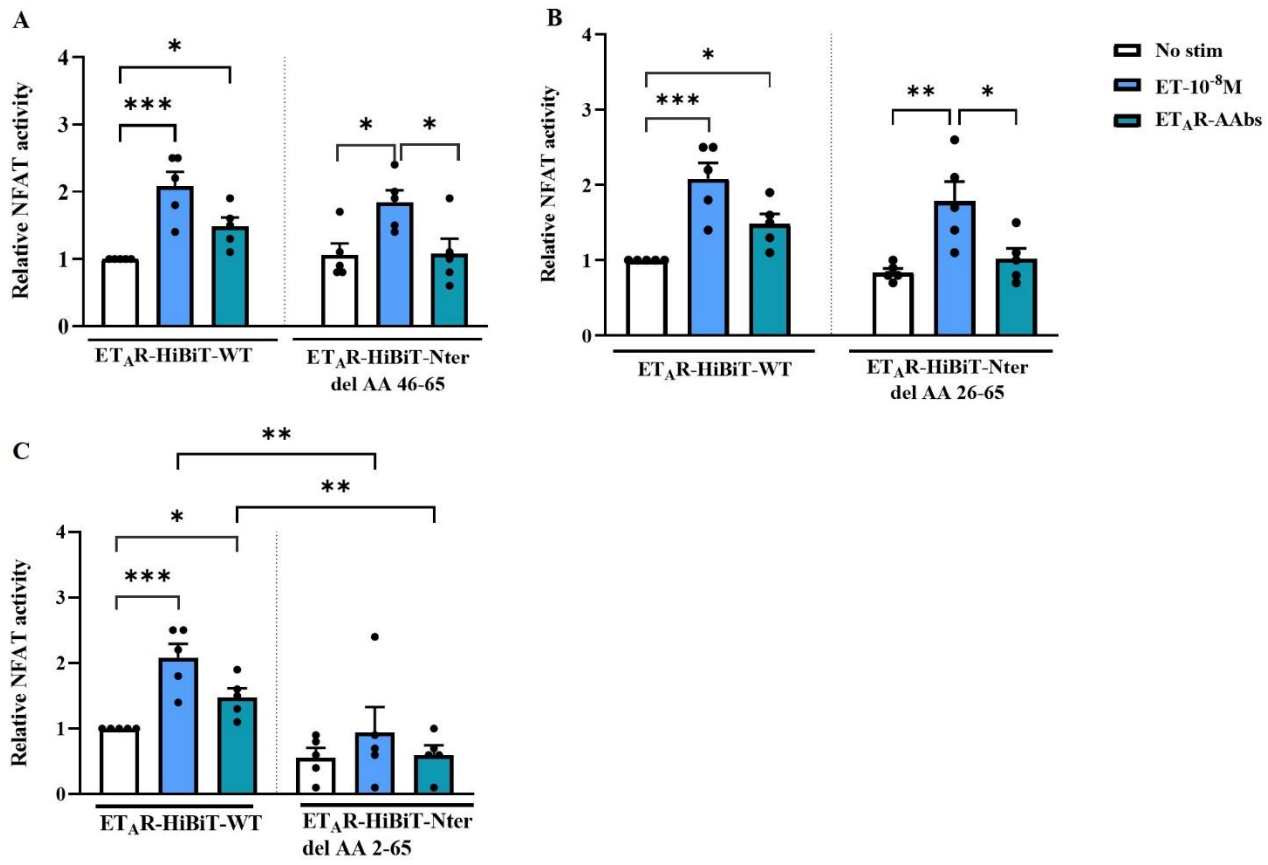

Figure 8: Deletion of ET_AR-Nter 2-25 reduces ET_AR-AAbs-mediated activation of $G_{q/11}$.
HEK 293 T were transfected with ET_AR-HiBiT-WT or with **(A)** ET_AR -HiBiT-Nter del AA 46-65 **(B)** ET_AR -HiBiT-Nter del AA 26-65 **(C)** ET_AR -HiBiT-Nter del AA 2-65. Transfected cells were stimulated with 1mg/mL of ET_AR-AAbs, and the activation of $G_{q/11}$ was monitored with NFAT-dependent luciferase luminescence. Data of five independent experiments are expressed as mean ± SEM, * $p<0.05$, **$p<0.01$, *** $p<0.001$ (Post-hoc tests after performing a Kruskal-Wallis test).

This demonstrates that ET_AR-Nter 2-25 is involved in the binding of ET_AR-AAbs to ET_AR triggering $G_{q/11}$ activation.

Regarding $G_{12/13}$ monitored with the transcription factor SRF, ET_AR-AAbs bound to the intact ET_AR significantly increased $G_{12/13}$ activation in the ET_AR-HiBiT-WT receptor compared to the non-stimulated control, as shown in the left part of the figures 9A-C. Again, the stimulatory effect of ET-1 10^{-8}M was stronger compared to ET_AR-AAbs. Conversely, deletion of AA 46-65 induced a significant greater activation of $G_{12/13}$ as seen in figure 8A, but the effect was lost with subsequent deletion of AA 26-65 and AA 2-65 (figure 9B-C).

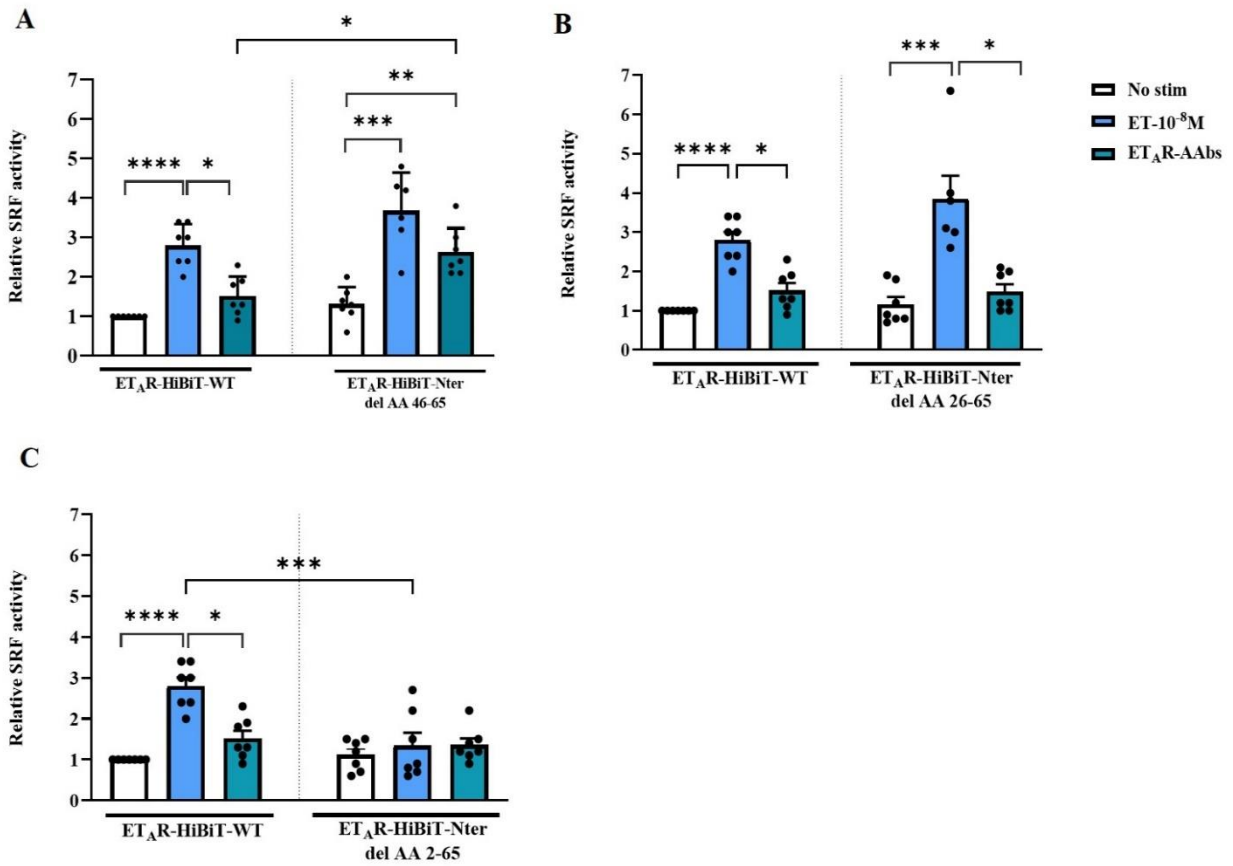

Figure 9: Deleting ET_AR-Nter AA 46-65 increases ET_AR-AAbs mediated activation of $G_{12/13}$.
HEK 293 T were transfected with ET_AR-HiBiT-WT or with **(A)** ET_AR-HiBiT-Nter del AA 46-65 **(B)** ET_AR-HiBiT-Nter del AA 26-65 **(C)** ET_AR-HiBiT-Nter del AA 2-65. Transfected cells were stimulated with 1mg/ml of ET_AR-AAbs and the activation of $G_{12/13}$ was monitored with SRF-dependent luciferase luminescence. Data of seven independent experiments are expressed as mean ± SEM, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ (Post-hoc tests after performing a Kruskal-Wallis test).

The results therefore demonstrate that removal of AA 46-65 from the intact ET_AR induces more a favorable receptor conformation of ET_AR-AAbs binding and activation of $G_{12/13}$. However, deletion of AA 26-45 and 2-25 represses the ET_AR-AAbs bound receptor conformation required for $G_{12/13}$ activation. Overall, ET_AR-AAbs bound to the intact ET_AR activate $G_{q/11}$ and $G_{12/13}$, but these effects are less strong than the natural ligand, ET-1. While ET_AR-Nter 2-25 involved in ET_AR-AAbs-mediated activation of $G_{q/11}$ and $G_{12/13}$, the loss of AA 46-65 promotes $G_{12/13}$ activation. Structural modeling of ET_AR-AAbs bound to ET_AR could provide insights into the interaction of ET_AR-Nter and ET_AR-AAbs to predict which individual AA are involved in $G_{12/13}$ activation.

4.3.2 ET_AR-Nter is not involved in ET_AR-AAbs-mediated activation of ERK 1/2

As ET-1 activates ERK 1/2 via binding to ET_AR, the effects of ET_AR-AAbs binding to ET_AR, as well as ET_AR-Nter involvement on ERK 1/2 activation were tested using the luciferase reporter plasmid SRE. As shown in the left part of the panels of figure 10, ET_AR-AAbs stimulated ERK 1/2 activation, albeit significantly less than ET-1. Deletions of AA 46-65, 26-65 and 2-65 from ET_AR-Nter produced no effect on ERK 1/2 activation (figure 10A-C).

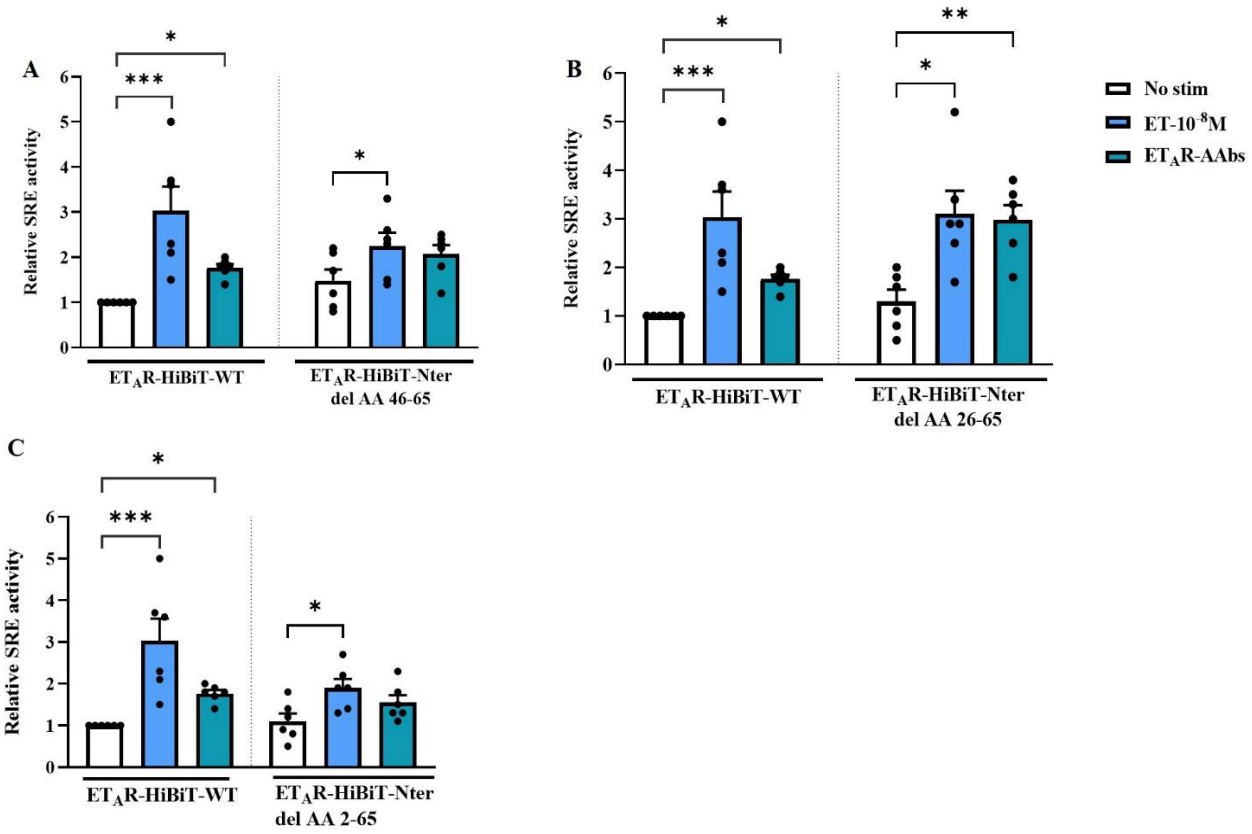

Figure 10: ET_AR-Nter does not affect ET_AR-AAbs-mediated activation of ERK 1/2.
HEK 293T were transfected with ET_AR-HiBiT-WT or with (A) ET_AR-HiBiT-Nter del AA 46-65 (B) ET_AR-HiBiT-Nter del AA 26-65 (C) ET_AR-HiBiT-Nter del AA 2-65. Transfected cells were stimulated with 1mg/ml of ET_AR-AAbs and the activation of ERK 1/2 was monitored with SRE-dependent luciferase production. Data of six independent experiments are expressed as mean ± SEM, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ (Post-hoc tests after performing a Kruskal-Wallis test).

This shows that ET_AR-Nter is not involved in ET_AR-AAbs-mediated activation of ERK 1/2.

4.4 **ET_AR-Nter AA 26-45 are involved in ET_AR-AAbs-mediated production of cAMP**

cAMP production is an important pharmacological target in SSc (51). Therefore, in addition to examining the effect of ET_AR-Nter in ET_AR-AAbs-mediated activation of G-proteins and ERK 1/2, the effect of cAMP after treatment with a concentration of ET-1 and ET_AR-AAbs was examined. Relative cAMP production after stimulation of PDE-blocked HEK 293 T was determined using a cAMP ELISA assay.

Binding of ET-1 and ET_AR-AAbs to the intact ET_AR significantly stimulated cAMP production as shown in the left part of figure 11A-C. However, ET_AR-AAbs elicited significantly more cAMP production than ET-1. In contrast, deletion of AA 46-65 produced no effect on ET_AR-AAbs and ET-1-mediated cAMP production, as shown in figure 11A.

Further deletion of AA 26-45 significantly reduced ET_AR-AAbs-mediated cAMP production but did not affect ET-1-induced cAMP production, as shown in figure 11B. Interestingly, further deletion of AA 2-25 abolished this effect and returned cAMP production to levels comparable to the ET_AR-HiBiT-WT after stimulation with ET_AR-AAbs, as shown in figure 11C. Of note, ET_AR-AAbs-induced cAMP production remained significantly stronger in all the deleted ET_AR-HiBiT-Nter constructs compared to non-stimulated cells.

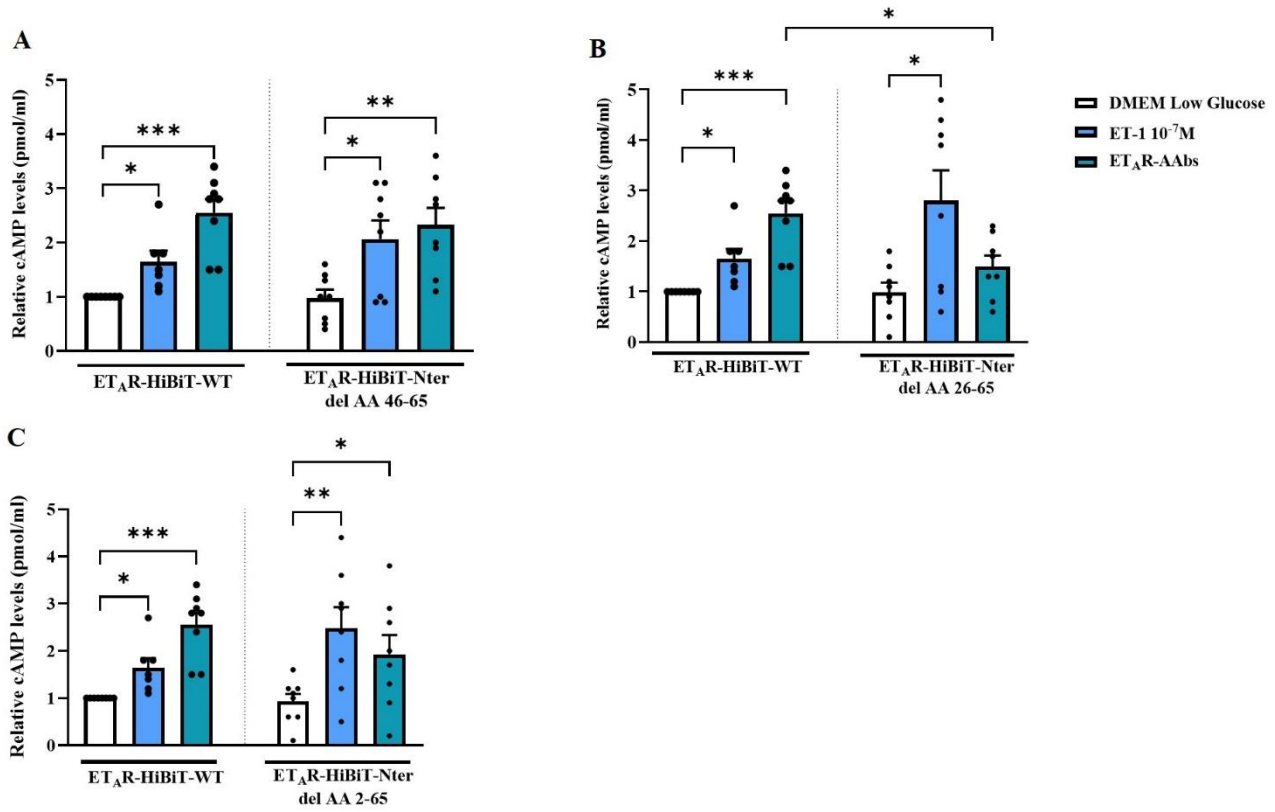

Figure 11: Loss of AA 26-65 decreases ET_AR-AAbs-mediated production of cAMP.
HEK 293T were transfected with ET_AR-HiBiT-WT or with **(A)** ET_AR-HiBiT-Nter del AA 46-65 **(B)** ET_AR-HiBiT-Nter del AA 26-65 **(C)** ET_AR-HiBiT-Nter del AA 2-65. 1mg/ml of ET_AR-AAbs and ET-1 10^{-7}M were stimulated, and the fluorescence from the ELISA assay was quantified. Data of eight independent experiments are expressed as mean ± SEM, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ (Post-hoc tests after performing a Kruskal-Wallis test).

The data suggest that different domains of ET_AR-Nter affect ET_AR-AAbs-mediated production of cAMP. The AA 26-45 domain is required for binding of ET_AR-AAbs to ET_AR, removal of which decreases cAMP production. Moreover, since deletion of the ET_AR-Nter also affected $G_{12/13}$ activation, it could be inferred that ET_AR-AAbs-mediated cAMP production is related to $G_{12/13}$.

5 Discussion

Systemic sclerosis (SSc), an autoimmune disease with localized and diffuse multi-organ involvement, is a major health challenge worldwide (1). The health challenge of SSc consists in the complexity of the disease process and the lack of specific treatment (1). Nevertheless, a breakdown in immune tolerance leading to the production of autoantibodies (AAbs) and damage to endothelial cells is observed and is responsible for many symptoms (1). Preclinical experimental studies indicate that dysregulation of the endothelin system leading to the overexpression of endothelin-1 (ET-1) or cognate receptor, endothelin type A receptors (ET_AR), is particularly involved in SSc pathogenesis (5, 6). Similarly, AAbs targeting ET_AR (ET_AR-AAbs) are considered a significant mediator of SSc inducing typical vasculopathologic features of inflammation, fibrosis and proliferation (58). Therefore, pharmacological inhibition of the endothelin axis is recommended as an effective therapeutic strategy (2, 6). However, the binding domains of ET-1 and ET_AR-AAbs are not precisely known. Given the exposure and distinctiveness of the extracellular amino (N)-terminal (Nter) domain of ET_AR (ET_AR-Nter) to the ligands, it is hypothesized that ET-1 and ET_AR-AAbs bind to the ET_AR-Nter domain in order to generate different dynamics of ET_AR signaling. Therefore, we investigated the role of the ET_AR-Nter in ET_AR-AAbs-mediated signaling dynamics, which differs from that of the natural ligand, ET-1. To validate the hypothesis, specific deletions of different amino acid (AA) segments of ET_AR-Nter, AA 2-25, 26-45 and 46-65 were generated. Plasmid constructs were tagged with the small protein HiBiT to reflect membrane expression of the receptor (67). We found that ET_AR-AAbs triggered ET_AR-mediated activation of $G_{q/11}$, $G_{12/13}$, and ERK 1/2 albeit less potently than the natural ligand, ET-1. However, ET_AR-AAbs increased cAMP production more than ET-1. The ET_AR-AAbs mediated ET_AR signaling dynamics was found to be influenced by the deletions of the different segments of the extracellular ET_AR-Nter. Apparently, AA 2-25 of the ET_AR-Nter is required for plasma membrane expression, binding and activation of $G_{q/11}$, $G_{12/13}$, ERK 1/2 and cAMP production for both ligands. However, while AA 46-65 and 26-45 affects ET_AR-AAbs mediated $G_{12/13}$ activation and cAMP production respectively, AA 26-45 affects ET-1-mediated $G_{12/13}$ activation. The data indicate that while ET-1 and ET_AR-AAbs induce ET_AR activation of $G_{q/11,}$ $G_{12/13,}$ and ERK 1/2 and cAMP production, but both ligands recognize different segments of ET_AR-Nter to bring forth different signaling dynamics.

5.1 Involvement of ET_AR-Nter AA 2-25 in ET_AR membrane expression and signaling

Membrane expression is a critical outcome of the intracellular trafficking and ligand-induced functionality of G-protein coupled receptors (GPCR). However, membrane expression is influenced by manipulating the structural and physical properties of the different domains of GPCR, which is exploited in pharmacology (70, 72, 73). In this current study, we find that deletion of ET_AR-Nter AA 2-25 increases the membrane expression of the receptor compared to wild type. Thus, deletion of AA 2-25 alters the structural integrity, resulting in accelerated trafficking and expression. As shown in the studies of α1D-adrenoceptor (α_{1D}-AR) (74) and cannabinoid receptor 1 (75), deletion of Nter AA promotes membrane expression by de-limiting misfolding motifs and ER retention. Although we have not monitored the intracellular transport mechanisms, we speculate that the deletion of AA 2-25 reduces the likelihood of endoplasmic reticulum (ER) retention and receptor misfolding, which impairs membrane expression, as the studies with the endothelin type B receptor (ET_BR) showed (36, 76). In the studies of ET_BR, the group of Schülein asserts that the first AA-25 contains the signal peptide sequences (SPS), whose truncation impairs receptors trafficking by limiting translocon gating and ER insertion (36). The group further demonstrated that deletion of ET_BR-Nter glutamic acid (Glu) ^{28} to tyrosine (Tyr) ^{54} (76) and the proteolysis of arginine (Arg) ^{64} and serine (Ser) ^{65} (34) after SPS tends to decrease membrane expression of ET_BR. Similarly, subsequent studies with the mutant corticotrophin-releasing factor receptor 1 lacking the SPS in the first 25 AA of the Nter show a significant decrease in receptor expression (77). This indicates that although ET_AR contain SPS in AA 2-25 as predicted by the computational study (78), deletion of the SPS can promote expression of the receptor. Similarly, in the case of the glucagon-like peptide-1 receptor, loss of the signal peptide sequence has been shown to significantly increase membrane expression (79, 80). This is because the SPS may only act to promote translocation and ER insertion (36), but may not be involved in post-translational events that are also required for cell membrane expression (79). In this case, N-linked glycosylation of Nter-AA residues might play a role in membrane expression (81). However, we found no evidence of the effect of deletion of the glycosidic residues asparagine (Asn) ^{26} contained in del AA 26-45 and Asn^{62} contained in del AA 46-65 on the ET_AR-Nter in the membrane expression, as in the study with the muscarinic-2 receptor (82) and the angiotensin II type 2 receptor (AT_2R) (83). But previous studies with ET-receptors in rat cerebral and atrial membranes show that deglycosylation affects the available binding sites suggesting a role in membrane expression (84). Given the limitation of not specifically digesting glycosylated residues in the study, future studies would need to be

conducted to elucidate the role of N-linked glycosylation in ET_AR membrane expression and functionality. In general, our results indicate that loss of putative SPS located in the first ET_AR-Nter AA2-25 enhances membrane expression necessary for the establishment of receptor functionality.

5.2 ET-1 activation of ET_AR signaling

As a typical GPCR, ET-1 binding to ET_AR induces a conformation leading to G-protein coupling and signaling dynamics relevant in vasculopathies including SSc (6, 16, 25). Reporter assays expressed in mammalian cell lines provide an efficient means of deciphering intracellular events following ligand stimulation of the expressed receptor (68). Our laboratory has optimized luciferase reporter systems for monitoring intracellular events from expressed receptors on various mammalian cell lines (46). These methods were applied in monitoring sub-specific G-protein activation using transcriptional reporter genes after dose-dependent stimulation with the ET-1. We observed that ET-1 dose-dependently increased the activation of $G_{q/11}$, $G_{12/13}$ and ERK 1/2. G-protein coupling is a fundamental step in receptor functionality (85). Thus, ET-1 bound to the intact ET_AR initiates conformational changes that recruit specific G protein for specific intracellular cascades and cellular responses. In the case of $G_{q/11}$, it is known that ET-1-induced ET_AR-mediated coupling leads to the production of pleiotropic calcium (Ca^{2+}) from inositol-1, 4, 5-triphosphate (IP_3) stores (17, 39). The intracellular accumulation of Ca^{2+} further mediates a variety of mechanisms leading to an adverse effect of vasoconstriction and activation of mitogenic signaling pathways (38). Similarly, experiments in vascular smooth muscle cells (VSMC) also show that ET_AR activated by ET-1 couples to $G_{12/13,}$ which in turn induces Ca^{2+}-dependent myosin-light-chain kinase (MLCK), a mediator of vascular contraction (39, 42). In addition, several studies have demonstrated the mitogenic effects of ET-1 via the production of ERK 1/2, a marker for cell proliferation (38, 86). However, different *in-vitro* and *in-vivo* models and G protein subtypes produce different activation profiles of ERK 1/2 (87). For example, in an adult rat ventricular myocytes, ET-1 induces ERK 1/2 via $G_{q/11}$ but not $G_{12/13}$ (88). However, in a rat tracheal smooth muscle cells, ET-1 stimulated ERK 1/2-mediated proliferation via the rat sarcoma virus protein homologue A (Rho-A), an effector of $G_{12/13}$ (89). Furthermore, studies with $G_{12/13}$ knockdown mice provided a direct association between $G_{12/13}$ activation and ERK 1/2 activation (90). In general, since ET-1 has been shown to activate ET_AR mediated G-protein coupling and activation of ERK 1/2, the dose-dependent effect of ET-1 may explain the sustained vascular effects of vasoconstriction and fibrosis observed in vasculopathies including SSc (5, 38, 54).

Therefore, the dose-dependent effect of ET-1 on ET_AR contributes to a fundamental understanding of vascular hemodynamics, the modulation of which can be a key therapeutic approach for vascular crises.

5.3 Regulation of ET_AR signaling selectivity by ET_AR-Nter AA 26-45 domain

The integrity of a receptor's structural conformation is important for signaling and functional relevance (85). Manipulation of GPCR structure provides a means for specific G protein selectivity and ligand binding dynamics (25, 85, 91). In particular, the extracellular loops, transmembrane domains, and the carboxyl terminus (Cter) are primarily targeted to induce signaling selectivity of endothelin receptors (24, 25). In this study, different parts of the ET_AR-Nter are shown to promote selective G-protein coupling. The deletion of AA 46-65 and AA 26-65 had no effect on the activation of $G_{q/11}$ and ERK 1/2. In contrast, deletion of AA 26-45 increased activation of $G_{12/13}$. This demonstrates that the interaction of ET-1 with AA 26-45 promotes the selectivity for $G_{12/13}$ signaling. This finding opens up the discussion on the role of Nter in ligand selectivity in the light of the sparse data on the structural behavior of ET_AR-Nter. It has previously been observed that mutagenesis of transmembrane residues, tyrosine (Tyr) ^{129} (92), as well as aspartate (Asp) ^{126} and Asp^{133} (29) influence ET_AR selectivity consistent with modelling data (21, 23). Consistent with the current study, Hashido's study also showed that deletion of different parts of ET_AR-Nter affects different ligand binding and signaling dynamics (93). In the Hashido study, deletions of ET_AR-Nter AA 25-70 and AA 25-49 expressed in the COS-7 cell line altered ET-1 binding dynamics (93). In addition, data from random mutagenesis and point mutations of ET_AR demonstrates ET-1-induced signaling selectivity (65). In a random mutagenesis study of ET_AR expressed in Chinese hamster ovary cells lacking the gene for glycine biosynthesis (CHO-KI) cells, point mutations of ET_AR-Nter Asn^{29}, aspartic acid $(Asp)^{35}$, lysine $(Lys)^{59}$ and methionine (Met) ^{65} reduced the $G_{q/11}$-mediated production of Ca^{2+} (65). In another study, substituted ET_AR-Nter mutants of Asp^{46} and arginine $(Arg)^{53}$ expressed in the COS-7 cell line also showed decreased ET-1 binding activity with concomitant decreased Ca^{2+} production (64). Furthermore, substitution of Asp^{35}, Lys^{59}, and cysteine (Cys) ^{69} from ET_AR-Nter reduced monomeric G13 coupling, while Met^{65} and Asp^{69} increased G13 coupling (65). In the case of angiotensin II type 2 receptor (AT_2R), the deletion of Nter AA 1-34 in the thermostabilized receptor also shows a ligand-specific effect (94). Again, deletion of Nter-AA 5-50 from AT_2R expressed in CV-1 in Origin, and carrying the SV40 genetic material-3 (COS-3) cell line, decreased angiotensin II (Ang II) ligand

binding activity (83). From the literature and current evidence, different parts of ET_AR-Nter assume different signaling outcomes that are relevant for specific pharmacological targeting. This further suggests that since the deletions of AA 26-45 and 46-65 had no effect on the activation of $G_{q/11}$ and ERK 1/2, activation of ERK 1/2 is associated with the consequent activation of $G_{q/11}$ as has been expressed in the literature (39, 87).

5.4 **Agonistic function of ET_AR-AAbs in ET_AR**

GPCR-AAbs produced by immune dysregulation or molecular mimicry elicit pathological consequences associated with autoimmune pathologies (7, 8, 59). In particular, ET_AR-AAbs have emerged as key players in the vascular crises observed in SSc and renal transplant rejection (8, 10). However, the agonistic potential, binding domain and mechanistic understanding are continuously explored. In the current study, ET_AR-AAbs bound to wild-type ET_AR were shown to activate $G_{q/11}$, $G_{12/13}$, ERK 1/2 and the production of cAMP, indicating an agonistic potential. Similarly, in the seminal study, Riemekasten *et al*., demonstrated that ET_AR-AAbs from SSc patients induce ERK 1/2 activation in human microvascular endothelial cells (HMEC-1) (10). Again, ET_AR-AAbs-induced $G_{12/13}$ and $G_{q/11}$ activation in G-protein specific yeast models and HMEC-1 cell lines was recently demonstrated (95). Catar *et al*., have also found the mechanistic relationship between endothelial proliferation and transcription of the transcription factor erythroblast 26 transformation specific-1 (Ets-1) in patients with scleroderma renal crisis (SRC) in addition to the agonistic value of ET_AR-AAbs (13). In addition, ET_AR-AAbs have also been shown to induce elevated levels of IL-8, CCL-18 and TGF-β in endothelial cells, suggesting a role in fibrosis and inflammation (11, 12). Immunized ET_AR-AAbs were also able to induce the expression of alpha-smooth muscle actin (α-SMA), a proliferative indicator, in a mouse model of pulmonary arterial hypertension (PAH) (15). Recently, patient ET_AR-AAbs have also been shown to further enhance the vasoconstrictor effects of ET-1 in SRC (14). Besides the agnostic potential of ET_AR-AAbs predominantly in SSc pathology, ET_AR-AAbs have been shown to orchestrate immune and non-immune cells trafficking in acute transplant rejection and cardiovascular pathologies (8). The aforementioned findings in addition to our results of ET_AR-AAbs-mediated activation of G-protein ($G_{12/13}$ and $G_{q/11}$) and ERK 1/2 and cAMP production expand the knowledge on agonistic value for which pharmacological targeting is beneficial.

5.5 Regulation of ET_AR-AAbs-mediated ET_AR signaling selectivity by ET_AR-Nter

GPCR-AAbs significantly stabilize GPCR conformation and signaling when bound to the extracellular regions (9). In particular, the extracellular loops 1-3 (ECL 1-3) are well recognized by GPCR-AAbs for receptor conformational stability and signaling selectivity (9). For example, angiotensin type 1 receptor-AAbs (AT_1R-AAbs) from kidney transplant patients bind to ECL-2 to initiate the downstream activation of ERK 1/2, a mediator of vascular proliferation and subsequent acute allograft rejection (62). Similarly, mutational studies revealed that ECL-2 is involved in AT_1R-AAbs-mediated endothelial cell proliferation via $G_{q/11}$ activation and transcription of nuclear factor of activated T cells (NFAT) (46). Furthermore, epitope mapping of ET_AR-AAbs from prostate cancer patients to the ECL of ET_AR demonstrated the involvement of ECL-2 in binding (96). Interestingly, in a mutational study involving ECL 1-3 of ET_AR, SSc-ET_AR-AAbs were shown not to be involved in $G_{q/11}$ and $G_{12/13}$ activation, suggesting a role for the Nter (95). Consequently, in this study it was found that different parts of ET_AR-Nter affect ET_AR-AAbs-mediated signaling selectivity of $G_{q/11}$, $G_{12/13,}$ ERK 1/2 activation and cAMP production. Remarkably, deletion of AA 2-25 and 46-65 influences the activation of $G_{q/11}$ and $G_{12/13}$ respectively, while deletion of AA 26-45 increases cAMP production. Thus, loss of AA 46-65 appears to stabilize the receptor conformation and promotes ET_AR-AAbs-induced $G_{12/13}$ activation, which is lost upon sequential deletion of AA 26-45 and 2-25. In this case, deletion of AA 26-45 destabilizes the receptor conformation required for production of cAMP. Similarly, deletion of AA 2-25 diminishes the receptor binding effect required for full activation of $G_{q/11}$. Interestingly, structural alteration of the ET_AR-Nter segments had no effect on the basal activity of the receptors contrary to the study of Wu, where change in ECL-3 resulted in constitutive activation (95). This further supports the evidence that, deletion of AA sequences affects ET_AR-AAbs-mediated signaling dynamics through interaction with different ET_AR-Nter segments, as revealed in the ligand binding studies with AA in the extracellular and transmembrane domains of the ET_AR and the antagonist bosentan (97). Conversely, ET_AR-Nter played no role in ET_AR-AAbs-mediated coupling of ERK 1/2 activation. However, since ET_AR-AAbs binding domains on ET_AR are unclear, modeling studies will provide further understanding of the ET_AR-AAbs binding domains on ET_AR. Nonetheless, the influence of ET_AR-Nter on the agonistic potential of ET_AR-AAbs requires strategies to mitigate possible effects on SSc pathology.

5.6 Mechanisms and functional consequences of ET_AR-AAb-mediated production of cAMP

cAMP homeostasis is essential for immune regulation and maintenance of vascular integrity (51, 98). Antibody induced production of cAMP could therefore offer a therapeutic advantage (51) In the current study, ET_AR-AAbs elicited higher production of cAMP than ET-1. However, the mechanistic understanding of ET-1-induced ET_AR-mediated cAMP production is complicated (38). Studies with VSMC show that ET-1 elicits cAMP production via $G_{\beta\gamma}$ rather than the known G_s-dependent pathway (47, 48). Again, in Henrietta Lacks (HeLa) and aortic smooth muscle cell lines overexpressing ET-1 receptors, ET-1 has been shown to activate protein kinase A (PKA), an effector of cAMP, independent of G_s (49). However, treatment of Chinese hamster ovary (CHO) cell line expressing ET_AR with guanosine triphosphate (GTP) demonstrate a direct relationship between G_s and cAMP production (99). Similarly, blocking $G_{q/11}$ coupling in a VSMC cell line also shows ET-1-induced G_s-dependent production of cAMP (47). However, since ET-1-induced cAMP levels are anti-inflammatory and anti-mitogenic in the normal vasculature, the production of cAMP could be a physiological response to vasculopathy (50, 51, 100). Thus, ET-1-induced pathological effects are counteracted by increased production of cAMP, which downregulates inflammatory genes and cell remodeling as observed in cardio-renal physiology (50, 100). Thus the stronger stimulatory effect of ET_AR-AAbs on cAMP suggests an autocrine effect in down-regulating the pathogenic effect of ET_AR-AAbs in SSc, hence a protective effect (98). Despite the interesting results derived from ET_AR-AAb-mediated production of cAMP, future studies are needed to examine the homeostatic balance with ET-1 to suggest whether ET_AR-AAb-mediated production is compensatory and therapeutic.

6 Conclusions and perspectives

In this study, both ET-1 and ET_AR-AAbs functionally activated ET_AR signaling, but ET_AR-AAbs evoked higher levels of cAMP. Remarkably, different parts of ET_AR-Nter contributed to differences in ET-1 and ET_AR-AAbs-mediated ET_AR signaling selectivity. The data suggest that the ET_AR-Nter is involved in ET_AR-AAbs-mediated signaling selectivity. This clinically important finding could be exploited in drug design. This work therefore provides preliminary information on the agonistic effect of ET_AR-AAbs and the role of ET_AR-Nterminus in influencing the dynamics and functionality of antibody-mediated signaling, a step towards the development of a novel therapeutic strategy.

However, this work was limited due to the absence of monoclonal antibodies targeting ET_AR and the lack of a crystal structure of ET_AR to better understand the structural and functional relationships between ET-1 and ET_AR-AAbs. Future studies will have to investigate the purification of ET_AR-AAbs to produce monoclonal antibodies and the modelling of ET_AR in complex with the antibodies to better elucidate the binding dynamics.

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Statutory Declaration

´´I, Michael Adu Gyamfi, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic The structural and functional activation of Endothelin-1 type A receptor (ET_AR) / Die strukturelle und funktionelle Aktivierung des Endothelin-1-Typ-A-Rezeptors (ET_AR), independently and without the support of third parties, and that I used no other resources and aids than those stated.

All parts, which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from the data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons. I have correctly marked all texts or parts that were generated in collaboration with other persons.

My contributions to any publication to this dissertation correspond to those stated in the joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; https://www.icmje.org/) on authorship. In addition, I declare that I shall comply with the regulations of Charité-Universitätsmedizin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me. ´´

Date: 30.June 2024 Signature:

Curriculum vitae

"My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection."

Publications

1. Moll G*, Luecht C*, Gyamfi MA*, da Fonseca DLM, Wang P, Zhao H, Gong Z, Chen L, Ashraf MI, Heidecke H, Hackel AM, Dragun D, Budde K, Penack O, Riemekasten G, Cabral-Marques O, Witowski J, Catar R. Autoantibodies from patients with kidney allograft vasculopathy stimulate a proinflammatory switch in endothelial cells and monocytes mediated via GPCR-directed PAR1-TNF-α signaling. Front Immunol. 2023 Oct 30.

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3. Zhao H, Chen Z, Fang Y, Su M, Xu Y, Wang Z, Gyamfi MA, Zhao J. Prediction of Prognosis and Recurrence of Bladder Cancer by ECM-Related Genes. J Immunol Res. 2022 Apr 12; 2022.

Acknowledgements

Certainly a great privilege to be counted as a student of the renowned Charité-Universitätsmedizin. Thank you Prof. Dr. Duska Dragun for accepting me into the Charité and your laboratory. Unfortunately you couldn't wait for this moment as you had to take the early lead out of the world. May your soul remain in peace!!

Thank you Prof. Dr. Klemens Budde for agreeing to continue supporting my work and the laboratory. In her doctoral thesis, Sumin Wu, a former student, described her as a small god, but I daresay she is a little above a small god. Dr. Aurélie Phillipe, words cannot fully express how you mean to me. You were not just a secondary supervisor but a great academic mentor and coach. I remain eternally grateful.

Furthermore, I would like to thank the post-docs in the laboratory of AG Budde; Dr. rer. nat. Rusan Catar, PD Dr. med. Angelika Kusch and Dr. Ing. Guido Moll for the guidance and support you have provided. I also thank former and current students, Dr. med. Christian Lücht, Dr. Ola Al Diab, Dr. med. Hongfan Zhao, Dr. med. Qing Li, Dr. Chen Lei, Dr. Dashan Wu, Ezgi Çpn, Jonas Bachmann and Pinchao Wang for the support and the great community we've built. I thank Mr. Marc Eigen for all the technical support.

In addition, my stay in Berlin and my studies would not have been possible without the scholarship from the German Academic Exchange Service (DAAD). I am grateful to the DAAD and the Ghanaian government for this great partnership. And also to the 2019 DAAD students for the various support systems that we have set up.

Finally, I would like to thank my family, especially my wife Eunice Mensah and the children for the sacrifices they make to ensure I can focus on this journey. To you, O Lord my God, Jesus Christ, thank you for building in me faith in possibilities when the journey was hard. With you it really is possible. Shalom!!

Confirmation by a statistician

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Bescheinigung

Hiermit bescheinige ich, dass Herr *Michael Adu Gyamfi* innerhalb der Service Unit Biometrie des Instituts für Biometrie und klinische Epidemiologie (iBikE) bei mir eine statistische Beratung zu einem Promotionsvorhaben wahrgenommen hat. Folgende Beratungstermine wurden wahrgenommen:

- Termin 1: 15.02.2023
- Termin 2: 23.02.2023

Folgende wesentliche Ratschläge hinsichtlich einer sinnvollen Auswertung und Interpretation der Daten wurden während der Beratung erteilt:

- Graphische Darstellung
- Kruskal-Wallis-Test
- Post-hoc-Tests

Diese Bescheinigung garantiert nicht die richtige Umsetzung der in der Beratung gemachten Vorschläge, die korrekte Durchführung der empfohlenen statistischen Verfahren und die richtige Darstellung und Interpretation der Ergebnisse. Die Verantwortung hierfür obliegt allein dem Promovierenden. Das Institut für Biometrie und klinische Epidemiologie übernimmt hierfür keine Haftung.

Datum: 24.02.2023 Name der Beraterin: Pimrapat Gebert

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