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IN VITRO STUDY OF THE EFFECTS OF ENDOTELIN-1: RELEVANCE TO THE PATHOGENESIS OF PULMONARY ARTERIAL HYPERTENSION IN SYSTEMIC SCLEROSIS

S.S.D. MED 09

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SOMMARIO

Introduzione: L'ipertensione arteriosa polmonare è una patologia grave con una sopravvivenza dei pazienti stimata del 68% a 1 anno, 48% a 3 anni e il 34% a 5 anni dalla diagnosi. Il ruolo dell'endotelina-1 è stato studiato in diverse condizioni patologiche tra le quali la sclerosi sistemica; nei pazienti affetti da sclerosi sistemica, l'endotelina-1 circolante è presente ad alto titolo suggerendo il suo coinvolgimento nello sviluppo del danno fibrotico e vascolare. L'endotelina-1 è, infatti, un peptide con attività pro-fibrotica, pertanto gli inibitori dei recettori endotelinici (ET_A e ET_B) possono esercitare un effetto anti-fibrotico. A livello vasale, inoltre, l'equilibrio degli effetti mediati dall'endotelina-1 sulle cellule muscolari lisce e sulle cellule endoteliali è responsabile dell'omeostasi del tono vascolare. Un altro interessante effetto dell'endotelina-1 sulle cellule endoteliali è l'induzione della produzione di aldosterone che, a sua volta, sembrerebbe incrementare i livelli di specie reattive dell'ossigeno. L'aldosterone è un mediatore chiave nel danno vascolare polmonare in risposta all'ipossia: l'attivazione del recettore per mineralcorticoidi dovuto all'aldosterone nelle cellule endoteliali *in vitro* è coinvolta nel rimodellamento vascolare e nella fibrosi in risposta all'ipossia e l'utilizzo di antagonisti aldosteronici attenua questi fenomeni.

Metodi: Per valutare l'espressione delle due isoforme recettoriali dell'endotelina-1 e del recettore per mineralcorticoidi, sono state effettuate analisi di reverse transcriptase-PCR, western blot e citofluorimetria. L'attivazione dei fibroblasti è stata valutata misurando con kit ELISA la quantità di collagene-1, TGF-β e PDGF nel terreno di coltura. La rilevazione della formazione di stress ossidativo intracellulare è stata valutata con citometria a flusso misurando la fluorescenza emessa dalla CM-H_2DCFDA dopo incubazioni lunghe o brevi con endotelina-1 e i suoi inibitori recettoriali o con l'aldosterone e il suo antagonista.

Risultati: Entrambi i recettori per l'endotelina-1 sono presenti sulla superficie dei fibroblasti polmonari umani e dei fibroblasti dermici normali umani disponibili in

commercio, ma anche su quella dei fibroblasti dermici ottenuti da donatori sani e da pazienti sclerodermici. L'espressione del recettore per mineralcorticoidi è stata rilevata nei fibroblasti polmonari umani, nei fibroblasti dermici sclerodermici e nelle cellule endoteliali polmonari umane mentre nei fibroblasti dermici normali umani e in quelli da donatori sani, la quantità di proteina recettoriale rilevata è stata più bassa che nelle altre cellule analizzate. Nei fibroblasti polmonari umani, in quelli dermici sclerodermici e nelle cellule endoteliali polmonari umane, l'endotelina-1 ha stimolato la produzione di specie reattive dell'ossigeno dopo incubazioni lunghe; tale produzione è stata inibita dall'utilizzo del bloccante per il recettore ET_B. L'aldosterone invece, ha stimolato la produzione di specie reattive dell'ossigeno dopo brevi incubazioni nei fibroblasti polmonari umani, in quelli dermici sclerodermici e nelle cellule endoteliali polmonari umane ma non nei fibroblasti dermici umani normali e in quelli dermici da donatori sani. Analoghi risultati sono stati ottenuti nei fibroblasti polmonari umani, in quelli dermici sclerodermici e nelle cellule endoteliali polmonari umane stimolandoli a lungo con endotelina-1 in presenza o assenza dell'inibitore del recettore per mineralcorticoidi.

Conclusioni: Questi risultati suggeriscono che l'endotelina-1 attiva i fibroblasti e la produzione di aldosterone, il vero responsabile dello stress ossidativo. L'inibizione della produzione di specie reattive dell'ossigeno dovuta all'utilizzo dell'antagonista del recettore ET_B, indica che tale recettore è implicato in questo processo. Sono state trovate differenze nella produzione di stress ossidativo dopo stimolazione con endotelina-1 tra i fibroblasti dermici ottenuti da donatori sani e quelli da pazienti sclerodermici. Inoltre, negli stessi tipi cellulari, sono state evidenziate differenze nella presenza del recettore per mineralcorticoidi.

ABSTRACT

Background: Pulmonary arterial hypertension is a devastating disease with an estimated patients' survival of 68% at 1 year, 48% at 3 years and 34% at 5 years from diagnosis. The role of endothelin-1 has been studied in different conditions including systemic sclerosis. In systemic sclerosis patients, circulating endothelin-1 is present at high levels, suggesting its involvement in the development of fibrotic and vascular damage. Indeed endothelin-1 is a potent pro-fibrotic peptide and inhibitors of endothelin-1 receptors (ET_A and ET_B) may exert an anti-fibrotic effect. The balance of endothelin-1 effects on smooth muscle cells and on endothelial cells is responsible for the homeostasis of vascular tone. Another interesting effect of endothelin-1 is the induction of aldosterone in endothelial cells that eventually results in the production of reactive oxygen species. Aldosterone is a key mediator of the pulmonary vascular injury response to hypoxia: mineralocorticoid receptor activation by aldosterone is involved in the remodeling/fibrosis response to hypoxia in endothelial cells *in vitro* and aldosterone antagonism attenuates these events.

Methods: Reverse transcriptase-PCR, western blot and cytofluorimetric analyses were performed to evaluate the expression of the two isoforms of endothelin-1 receptors and mineralocorticoid receptor. Activation of fibroblast was evaluated measuring the amount of collagen-1, TGF-β and PDGF with ELISA kits. The detection of intracellular oxidative stress formation was measured with flow cytometry using fluorescence of CM-H_2DCFDA after long or short incubations with endothelin-1 and its receptors inhibitors or with aldosterone and its antagonist.

Results: Both endothelin-1 receptors are present in commercially available human pulmonary fibroblasts and normal human dermal fibroblasts, human dermal fibroblasts from healthy donors and scleroderma human dermal fibroblasts. Mineralocorticoid receptor expression was detected in human pulmonary fibroblasts, scleroderma human dermal fibroblasts and human pulmonary artery

endothelial cells while in normal human dermal fibroblasts and human dermal fibroblasts from healthy donors the amount of this receptor was lower than the other cells analyzed. In human pulmonary fibroblasts, scleroderma human dermal fibroblasts and human pulmonary artery endothelial cells, endothelin-1 stimulates the production of reactive oxygen species after a long incubation. In addition, the production of oxygen radicals is inhibited by the use of ET_B antagonist. Interestingly, aldosterone stimulates the production of reactive oxygen species after a short incubation in human pulmonary fibroblasts, scleroderma human dermal fibroblasts and human pulmonary artery endothelial cells, but not in normal human dermal fibroblasts and human dermal fibroblasts from healthy donors. The same results were obtained by human pulmonary fibroblasts, scleroderma human dermal fibroblasts and human pulmonary artery endothelial cells stimulated with endothelin-1 for long time with mineralocorticoid receptor inhibitor.

Conclusions: These results suggest that endothelin-1 activates fibroblasts and the production of aldosterone, the true responsible for oxidative stress. The inhibition of reactive oxygen species production using the ET_B antagonist indicates that the ET_B receptor is implicated in this process. Moreover we found differences between fibroblasts from healthy donors and from scleroderma patients in oxidative stress production after endothelin-1 stimulation. Finally we found differences in mineralocorticoid receptor presence in the same cell types.

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1. INTRODUCTION

1.1 Pulmonary arterial hypertension

Pulmonary hypertension (PH) is a disorder that may complicate different cardiovascular and respiratory diseases. The definition of PH is purely hemodynamic: detection of mean pulmonary artery pressure (PAPm) ≥25 mmHg at rest measured by right heart catheterization (RHC) [1]. The normal PAPm at rest is 14±3 mmHg with an upper normal limit of 20 mmHg while values between 21 and 24 mmHg have an unclear clinical significance [1, 2]. Pulmonary arterial hypertension (PAH) is defined as a condition hemodynamically characterized by the presence of pre-capillary PH, with a pulmonary artery wedge pressure (PAWP) ≤15 mmHg and a pulmonary vascular resistance (PVR) >3 WU in the absence of other causes of pre-capillary PH [1]. Combining parameters as PAP, PAWP, PVR, cardiac output and pressure gradient (DPG) it is possible to have different hemodynamic definitions of PH (Table 1) [1, 3].

The clinical classification of PH takes into account multiple clinical conditions sharing similar presentation, hemodynamic characteristics and treatment strategy. The first classification of PH was proposed in 1973. In 2008, the 4th World Symposium on PH held at Dana Point (California, USA) revised previous classifications [4] and in 2013, the 5th World Symposium on PH, held in Nice (France), slightly modified the Dana Point classification, (Table 2) maintaining the division into 5 subgroups [5]: 1) pulmonary arterial hypertension (PAH); 2) pulmonary hypertension due to left heart disease; 3) pulmonary hypertension due to lung diseases and/or hypoxia; 4) chronic thromboembolic pulmonary hypertension (CTEPH); 5) pulmonary hypertension with unclear multifactorial mechanisms.

The diagnosis of PH needs a comprehensive set of hemodynamic investigation to confirm the clinical suspicion and requires expertise in cardiology, imaging and respiratory medicine. A multidisciplinary team is

particularly important to identify patients with different causes of PH; for this reason, a diagnostic algorithm has been proposed (Figure 1) [6]. PH incidence data at the global level are poor because PH is present in many conditions and it can be asymptomatic or with few non-specific symptoms in the early stages [5]. Focusing our attention on PAH, in Europe its prevalence and incidence are in the range of 15–60 subjects per million population and 5–10 cases per million per year, respectively [7]. Therefore PAH is considered a rare disease with an estimated patients' survival of 68% at 1 year, 48% at 3 years and 34% at 5 years from diagnosis [8].

The treatment of PAH patients can be divided into three main steps [9]: 1) general measures (physical activity, elective surgery, avoidance of pregnancy and post-menopausal hormonal therapy, infection prevention, psychosocial support); 2) supportive therapy (oral anticoagulants, diuretics, oxygen, cardiovascular drugs, iron substitution); 3) specific drugs therapy (calcium channel blockers, endothelin receptor antagonists, phosphodiesterase type 5 inhibitors and guanylate cyclase stimulators).

Each of the five PH groups is characterized by a specific histological and morphological pattern [10, 11]. In PAH distal sections of pulmonary artery with a diameter of less than 500 μm (40-300 μm) are involved. The principal element is a pathophysiological vascular remodeling (Figure 2) with a strong increase in thickness of the muscular media, the proliferation of the intima (neo-intima formation), eccentric or concentric fibrotic changes, thickening adventitia with perivascular inflammatory infiltrates, plexiform lesions with a classic look to onion-layering (30-60% of patients) and the presence of small thrombi (40-50% of patients). It is important to underline that the veins are never affected by these structural changes while the arterioles and the small arteries are interested by a major reduction in their lumen [12, 13].

The mechanisms that contribute to PAH are complex and not clearly defined. It is believed that etiology is multifactorial including genetic, autoimmune, biochemical and environmental factors [14]. However there are three mechanisms which, taken together, produce the increase in pulmonary

vascular resistance: the vasoconstriction, the remodeling of the vascular wall and *in situ* thrombosis [15]. Therefore the increase of the vascular resistance is determined by functional and anatomical phenomena (Figure 3) [16].

1.2 Systemic sclerosis

Systemic sclerosis (SSc), or scleroderma, is a rare autoimmune disease of unknown origin, characterized by immune dysregulation, vasculopathy and multi-organ fibrosis. Severe and diffuse endothelial cell (EC) damage and fibroblasts activation with overproduction and accumulation of collagen and other extracellular matrix proteins lead to skin thickening and fibrosis of the affected organs [17]. Three are the main pathogenetic mechanisms involved in this disease: 1) vascular dysfunction and injury; 2) activation of the immune system; 3) increased collagen secretion by fibroblasts.

The standard classification criteria for SSc have been recently revised by the American College of Rheumatology (ACR) and the European League Against Rheumatisms (EULAR). These criteria identify three different forms of the disease [18, 19]: 1) localized cutaneous scleroderma; 2) SSc; 3) overlap syndromes. Localized scleroderma, more common in childhood, is characterized only by skin involvement and rarely the disorder is systemic [20, 21]. SSc is characterized by the involvement of skin and different internal organs. Considering the cutaneous extension of fibrosis, SSc can be defined as: limited SSc (lSSc) with skin thickening of the face and distal part of the limbs; diffuse SSc (dSSc) characterized by a cutaneous thickening that also involves the proximal portions of the limbs and often the trunk and abdomen. The diagnosis of overlap syndrome can be performed when the criteria for diagnosis of SSc and of another autoimmune disease (systemic lupus erythematosus, Sjögren's syndrome, rheumatoid arthritis and other types of connectivitis) are present.

The epidemiology of SSc is difficult to estimate because of the differences in the geographical area, the disease definition and the method of case ascertainment [22]. The SSc prevalence in global population is estimated to be 50-300 cases per million inhabitants with an incidence ranging of 2.3-22.8 cases per million per year [23]. The disease has a female/male ratio varying from 3:1 to 14:1 according to different studies with its onset between the third/fifth decade of life. The higher incidence has been reported in a Choctaw Native American group in Oklahoma [24, 25].

SSc etiology is not fully known yet. Genetic and environmental factors play an important role in the etiopathogenesis of the disease [26-28] resulting in the three fundamental aspects of the disease: 1) the vasculopathy; 2) the alterations of cellular and humoral immune response; 3) the excessive deposition of collagen and other extracellular matrix proteins.

The primary event in SSc pathogenesis is the vascular damage that is a systemic process which involves the small blood vessels evolving in fibrosis [29-31]. The first cells involved in scleroderma damage are the ECs with an increased expression of adhesion molecules responsible for: leukocytes recruitment and activation; basal lamina thickening; pericytes, fibroblasts and smooth muscle cells (SMCs) proliferation; platelet adhesion and activation; formation of perivascular infiltrate [29, 32, 33].

1.3 Endothelin-1

Endothelin-1 (ET-1) is a small peptide of 21 amino acids with a hydrophobic C-terminus and two cysteine bridges at the N-terminus [34]. It is mainly a vasoconstrictor mediator but other effects have been described. Indeed it plays a role in inflammation, cell adhesion, fibrosis and angiogenesis [35]. ET-1 was isolated from cultured porcine aortic ECs in 1988 and 1 year later, two structurally related peptides, differing by two and six amino acids, were identified

and termed endothelin-2 (ET-2) and endothelin-3 (ET-3) respectively (Figure 4) [34, 36]. Nowadays four isoforms of endothelin are known: ET-1, ET-2, ET-3 and ET-4. ET-1 is produced by a large number of cells (SMCs, fibroblasts, myofibroblasts, mastocytes, monocytes/macrophages, polymorphonucleate cells, dendritic cells and others); ET-2 is mainly produced by intestinal and renal cells; high levels of ET-3 are found in central nervous system; little is known about ET-4 (Table 3). It has been shown that ET-1 plays an important role in cardiovascular, renal and pulmonary pathologies (PH included), carcinogenesis and fibrosing diseases (including SSc) [34, 37-39].

The gene encoding for ET-1 is located on the short arm of chromosome 6 and the activation of this gene is regulated by transcription factors as c-fos, c-jun, NFkB-1, AP-1 and GATA-2. PreproET-1 undergoes post-traductional modifications by two proteases (furin-like endopeptidase) becoming PreET-1 and then BigET-1. In the extracellular compartment BigET-1 is converted in ET-1 (Figure 5) [34]. The production of this peptide is stimulated by hypoxia, exposure to cold, low wall tension, angiotensin II, growth factors and cytokines [37, 38].

ET action is mediated by its three G-protein-coupled receptors: ET_A, ET_B and ET_C. ET_A binds ET-1 and ET-2 with higher avidity than ET-3 but it is more specific for ET-1. ET_B binds ET-1, ET-2 and ET-3 with the same specificity. Little is known about ET_C. ET receptors are expressed on various cell types. The type of receptor bound, the cell type stimulated and the tissue conditions modulate the different effects of this peptide [34, 35, 39, 40].

ECs express exclusively ET_B, while SMCs express predominantly ET_B. Generally the balance of ET-1 actions on SMCs and ECs is responsible for the homeostasis of vascular tone: on SMCs, ET_A is responsible for contraction due to production of inositol 1,4,5-trisphosphate (IP_3); on ECs, ET_B modulates the contraction of SMCs with production of nitric oxide (NO) (Figure 6) [34]. Alteration of this balance causes vasoconstriction. Fibroblasts express both ET_A and ET_B receptors and ET-1 can activate the production of collagen and induces

the expression of ICAM-1. Indeed it may stimulate the transdifferentiation of fibroblasts into myofibroblasts [41, 42]. The ET-1 induced collagen production by fibroblasts is not reduced by the selective antagonism of ET_A or ET_B, while it is reduced by combined ET_A/ET_B antagonism that reverts the fibrotic fibroblasts phenotype to normal phenotype. This would suggest the presence of cross-talk between the two receptors [41, 43].

The role of ET-1 has been studied in SSc and PAH because SSc and/or PAH patients present elevated ET-1 levels suggesting the involvement of this peptide in the development of fibrotic and vascular events [44-46]. Indeed the ET_A/ET_B antagonist Bosentan has been used as a novel anti-fibrotic drug contrasting the potent pro-fibrotic effect of ET-1 [47]. In a rat model, ET-1 induces the redistribution of endothelial nitric oxide synthase (eNOS) uncoupling from the plasma membrane to the mitochondria in pulmonary arterial endothelial cells (PAECs); also ET-1 disturbed carnitine metabolism, resulting in the attenuation of mitochondrial bioenergetics. However, ATP levels are unchanged due to a compensatory increase in glycolysis [48]. Another interesting aspect of high levels of ET-1 is the production of aldosterone in ECs that eventually results in the production of reactive oxygen species (ROS). ROS block the interaction between ET_B and eNOS reducing the Cys405 in ET_B, important for the activation of eNOS [35].

1.4 Aldosterone

Aldosterone is the main steroid mineralocorticoid hormone synthetized in the glomerular zone of the adrenal cortex in response to sodium depletion or hyperkaliemia but a local production of this hormone may also occur in peripheral tissue; it is the last product of the renin-angiotensin-aldosterone system (RAAS) activation [49]. The pivotal role played by RAAS is the maintenance of sodium and water balance through its action on kidney [50]. In response to a decrease in circulating blood volume (as blood loss, dehydration, ventricular pump failure)

the RAAS regulates intravascular volume releasing renin into circulation. The juxtaglomerular cells of the kidney release renin to increase angiotensin II that increases blood pressure by stimulating aldosterone production from the adrenal gland (Figure 7) [51].

Aldosterone has different genomic and non-genomic effects. The genomic effects are mediated by the intracellular mineralocorticoid receptor (MCR) and they include increasing in protein synthesis, inflammation and fibrosis. The genomic effects can be divided into classical and non-classical effects played in the renal distal convoluted tubule level and in different organs respectively [52-54]. The little known non-genomic effects are mediated by a membrane receptor of unknown nature and they include increasing in tyrosine phosphorilation, activation of inositol phosphate, increasing in sodium/hydrogen exchange and SMCs alkalinization. The non-genomic effects are principally explicates in the vessels [52, 55, 56].

There is a clear evidence that the RAAS is activated in PAH. Preclinical studies performed in a rat model with PH demonstrated an increase of angiotensin-converting enzyme (ACE) expression with an approximately 50% increase in ACE activity in pulmonary vessels [57]. Nowadays different studies suggest aldosterone as a mediator of the cardiopulmonary vascular phenotype in PAH; indeed high aldosterone levels have been detected in experimental models of PAH and in patients affected by the disease. This is a consequence of increased adrenal production of aldosterone but also of extra-adrenal synthesis of the hormone that has been identified in the pulmonary vasculature [35, 58, 59].

ET-1, which is upregulated in PAH, is known to induce the production of aldosterone. Indeed hyperaldosteronism in PAH is confirmed [60]. These persistently elevated aldosterone levels activate MCR that is expressed in vascular and cardiac cells contributing to vascular remodeling, altered vascular reactivity and cardiac dysfunction. In the PAH rat monocrotaline model, ET-1 levels in plasma and lungs are increased; this is associated with high aldosterone levels in the same fluid/tissue. ET-1 is able to stimulate extra-adrenal pulmonary aldosterone synthesis increasing pulmonary endothelial expression of aldosterone

synthase (CYP11B2). In this model the mineralocorticoid receptor antagonists spironolactone or eplerenone decrease the number of muscularized vessels and limit vascular collagen deposition [35].

1.5 Reactive oxygen species

ROS are chemically reactive molecules containing oxygen constantly formed in the body and removed by antioxidant defenses preventing their formation or repairing the damage that they induce [61]. ROS, but also reactive nitrogen species (RNS), play a dual role having both deleterious and beneficial effects. ROS and RNS are generated by regulated enzymes as NO synthase (NOS) and NADPH oxidase isoforms respectively. Low/moderate concentrations of ROS/RNS have beneficial effects in physiological processes, for example in defense against infectious agents, in cellular signaling pathways and in induction of a mitogenic response. In contrast, overproduction of ROS results in oxidative stress, a deleterious process responsible for cell damage [62]. The paracrine action of endothelium-derived NO controls vascular tone, inhibits platelet function, prevents leukocytes adhesion and reduces intima proliferation. Inactivation and/or reduced synthesis of NO can promote vasospasm, thrombosis, vascular inflammation and SMCs proliferation [63].

Pulmonary endothelial ROS have been implicated in PAH and have been shown to disrupt NO-dependent vasodilatory signaling pathways to promote pulmonary vasoconstriction, muscularization of pulmonary arterioles and perivascular fibrosis [64, 65]. Activation of eNOS mediated by ET_B results in a production of endogenous NO in pulmonary tissue [66] but how ROS decrease the production of NO in PAH is not clear. ET_B contains an intracellular cysteine-rich region near its C-terminus (Figure 8) [67], in this domain there is Cys405, demonstrated to be a cysteinyl thiol that regulates ET_B signal transduction [68]. In a model proposed by Maron et al. (Figure 9) [35], the oxidative modification of Cys405 by aldosterone-induced ROS disables ET_B-eNOS activation promoting pulmonary vascular dysfunction in pulmonary artery endothelial cells (HPAECs).

2. AIM OF THE PROJECT

ET-1 plays a pivotal role in the three major aspects involved in the pathogenesis of SSc and PAH: vasoconstriction, fibrosis and inflammation. At the moment we know that ET-1 is responsible of dysregulation of vascular tone and, consequently, of endothelial cell damage. Indeed ET receptors are expressed on the majority of cells involved in SSc and PAH, such as ECs, SMCs and fibroblasts; for this reason ET_A/ET_B antagonists are used in treatment of SSc patients with recurrent ischemic digital ulcers and/or PAH with beneficial effects on fibrosis and vasoconstriction. A recent study has revealed that ET-1 is able to increase the aldosterone production in pulmonary ECs and that this hormone is responsible for the formation of ROS.

The aim of this PhD project was to clarify the role of ET-1 in the pathogenesis of PAH and to investigate the effects of this molecule on pulmonary vascular cells analyzing its effects on lung fibroblasts and ECs. We have also studied the effects of ET-1 on fibroblasts derived from skin of SSc patients and healthy donors. This study was based on the hypothesis that ET-1 could have a fundamental role on ROS production and fibroblasts activation. For this reason we have analyzed the effects of ET-1 stimulation on fibroblasts and oxidative stress production; moreover we have investigated the link between ET-1 and aldosterone.

3. MATERIALS AND METHODS

3.1 Cell cultures and treatments

This study was performed using human pulmonary fibroblasts (HPFs), normal human dermal fibroblasts (NHDFs) and human pulmonary artery endothelial cells (HPAECs) (PromoCell, Heidelberg, Germany). Moreover dermal fibroblasts from healthy donors (HDFs) and from SSc patients (SSc HDFs) obtained by skin biopsy were used. Cells were grown to confluence at 37°C and 5% CO_2 by using fibroblast growth medium 2 and endothelial growth medium (PromoCell) for the commercial lines while in DMEM (ThermoFischer Scientific/Gibco, Waltham, MA, USA) +10% FBS (Sigma-Aldrich, St. Louis, MO, USA) +1% penicillin streptomycin (pen-strep; ThermoFischer Scientific/Gibco) for the cells from skin biopsy. Cells were passaged by using accutase (Millipore, Billerica, MA, USA) and experiments were performed on cells from passages 4 to 10.

ET-1 (100 nM) (Tocris Bioscience, Bristol, UK) and BQ123 (1.5 μM) (Sigma-Aldrich), a selective ET_A endothelin receptor antagonist, were dissolved in RPMI (Lonza, Basel, Switzerland); BQ788 (1.5 μM) (Sigma-Aldrich), a selective ET_B endothelin receptor antagonist, aldosterone (100 nM) (Sigma-Aldrich) and the MCR inhibitor spironolactone (10 μM) (Sigma-Aldrich) were dissolved in DMSO (Sigma-Aldrich).

3.2 HDFs isolation

HDFs were isolated from skin biopsies of healthy donors and of limited cutaneous SSc patients who kindly offered a skin sample for research purposes after written informed consent. Biopsy samples (0.6 cm of the forearm skin) were placed immediately in DMEM +1% pen-strep. In sterile hood, the skin samples were transferred in 1 mL digestion medium (DMEM +20% FBS +0.25%

collagenase type I +0.05% DNAse I +1% pen-strep) than they were placed overnight in 37°C tissue culture incubator (collagenase type I and DNAse I were purchased from Sigma-Aldrich). Next day, the samples were vortexed for 20 seconds and, in sterile hood, 7 mL of culture medium (DMEM +20% FBS +1% pen-strep) were added. Then entire contents were transferred in T75 tissue culture flasks (BD Bioscience/Falcon, San Josè, CA, USA) that were placed in 37°C tissue culture incubator for 72 hours. On day 6, 7 mL of culture medium (DMEM +10% FBS +1% pen-strep) were added. After cells were passaged for the first time, the culture medium used was DMEM +10% FBS +1% pen-strep.

3.3 RNA isolation and RT-PCR

HPFs, NHDFs and HPAECs were cultured in 6-wells plates (BD Bioscience/Falcon) and were stimulated by ET-1 for 24 hours. Using TRI Reagent (Sigma-Aldrich) total RNA was obtained and then used for retrotranscription in cDNA. Amplification of cDNA was performed using AmpliTaq Gold PCR MasterMix (Applied Biosystems, Foster City, CA, USA) and GenAmp PCR System 9700 thermocycler (Applied Biosystems). The following primers (Sigma-Aldrich) were used for ET_A, ET_B, MCR and α-SMA detection:

ET_A:	forward	5'-ATGCACAACTATTGCCCACA-3'
	reverse	5'-GGACAGGATCCAGATGGAGA-3'
ET_B:	forward	5'-GCACATCGTCATTGACATCC-3'
	reverse	5'-CAGAGGGCAAAGACAAGGAC-3'
MCR:	forward	5'-AGGCTACCACAGTCTCCCTG-3'
	reverse	5'-GACTGGAGATTTTACACTGC-3'
α-SMA:	forward	5'-GGAATCCTGTGAAGCAGCTC-3'
	reverse	5'-GAAGGAATAGCCACGCTCAG-3'

Vimentin was used as internal control.

3.4 Western blot analysis

NaCl, TRIS, Tween 20, $MgCl_2$, β-mercaptoethanol, glycine, glycerol, bromphenol blue, sodium dodecil sulphate (SDS), 30% acrylamide/bis solution, methanol, ammonium persulfate (APS) were purchased by Sigma-Aldrich; temed was purchased from GE Healthcare Life Biosciences (Little Chalfont,UK); Dulbecco's phosphate buffered saline (DPBS) was from Lonza.

Western blot analyses were performed to confirm the expression of ET_A and ET_B receptors on cell surface but also to evaluate the presence of MCR and CYP11B2 in the intracellular space. Total protein lysate was obtained using RIPA buffer +1% protease inhibitors mix (Roche, Penzberg, Germany). Equal amounts of protein samples (20 μg) were separated by electrophoresis with 10% acrylamide gel under reducing conditions and transferred to nitrocellulose membrane (GE Healthcare Life Biosciences/Amersham Biosciences, Little Chalfont, UK). Membranes were then incubated in blocking buffer containing 5% non-fat dry milk powder (Sigma-Aldrich). ET_A, ET_B, MCR and CYP11B2 were detected incubating the membrane with 1:100 polyclonal primary antibody anti-ET_A (Acris Antibodies, GmbH, Herford, Germany) or 1μ/ml polyclonal anti-ET_B (Lifespan Biosciences, Seattle, WA, USA) or 1:500 polyclonal primary antibody anti-MCR (Abcam, Cambridge, United Kindom) or 1:60 polyclonal primary antibody anti-CYP11B2 (Abcam) overnight at 4°C. 1:100 polyclonal primary antibody anti-β-actin (Sigma-Aldrich) was used as internal control. Membranes were then incubated with a 1:3000 anti-rabbit IgG-HRP for ET_A (GE Healthcare, Freiburg, Germany) or a 1:3000 anti-sheep IgG-HRP for ET_B (Bethyl Laboratories, Inc. Montgomery, TX, USA) or 1:1000 anti-rabbit IgG-HRP (Santa Cruz Biotechnology, Dallas, TX) for MCR, CYP11B2 and β-actin for 1 hour at room temperature. The detection was performed using the ECL detection system (GE Healthcare Life Biosciences/Amersham Biosciences) and the Image Quant Las Mini 4000 Digital Imaging System (GE Healthcare Life Sciences) while densitometric analysis was carried out using the ImageQuant TL software (GE Healthcare Life Sciences).

3.5 Flow cytometry

Flow cytometry was used to detect the presence of ET_A and ET_B receptors on cell surface and to evaluate the oxidative stress induced by ET-1 or aldosterone. Samples were acquired with a FACSCanto II cytometer (BD Bioscience) and data were analyzed by FlowJo software (Treestor, Ashland, OR, USA).

Cells were incubated with anti-ET_A or anti-ET_B polyclonal primary antibody used also for western blot analyses and stained with R-Phycoerythrin (PE) secondary monoclonal anti-rabbit or anti-sheep IgG (R&D Systems, Minneapolis, MN, USA) respectively; cells incubated only with secondary antibodies were used as negative controls.

The detection of intracellular oxidative stress formation was based on the oxidation of 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA; Invitrogen, Oregon, USA) 50 μM dissolved in DMSO to yield an intracellular-trapped fluorescent compound. This molecule is extremely sensitive to changes in the redox state of cells and can be used to follow changes in ROS over time. Its fluorescence was measured with flow cytometry after short (40 minutes) or long (24 hours) stimulation with ET-1 in cells grown with 10% human serum (Invitrogen) and after incubation with CM-H_2DCFDA for 30 minutes. To evaluate the role of individual receptor engagement, cells were also pre-incubated with BQ123 and/or BQ788 for 30 minutes. ROS production was also evaluated after short stimulation with aldosterone (40 minutes) and after pre-incubation with spironolactone (30 minutes). In addiction, cells underwent long ET-1 stimulus (24 hours) and concurrently incubated with spironolactone. In every experiment H_2O_2 was used as internal positive control and washes were performed using Hank's balanced salt solution (HBSS; ThermoFischer Scientific/Gibco).

3.6 ELISA kit

The HPFs and NHDFs supernatant concentrations of collagen-1, TGF-β and PDGF were measured by enzyme-linked immunosorbent assay (ELISA) before ad after 24- or 48-hour stimulation with ET-1. Collagen-1 ELISA kit was purchased by BlueGene Biotech (Shanghai, China) while TGF-β and PDGF ELISA kits by R&D Systems. The samples preparation and the assay were performed following the manufacturer's recommendations. The enzymatic reaction optical densities were determined reading the plates at 450 nm with TECAN Sunrise III (Tecan, Männedorf, CH).

3.7 Statistical analysis

All statistical analyses were performed using GraphPad Prism version 5 (GraphPad Software Inc., La Jolla, CA, USA). Data with a normal distribution were analyzed with Student's t-test and were expressed as a mean ± standard deviation. A difference between groups with a $p<0.05$ was considered statistically significant.

4. RESULTS

4.1 ET-1 receptors

The presence of the two ET receptor isoforms (ET_A and ET_B) in HPFs was carried out at two levels: at transcriptional level evaluating the presence of ET_A/ET_B mRNA by RT-PCR; at protein level by flow cytometry and western blot analysis in order to confirm the expression of ET receptors on the cell surface. The potential expression differences in the same cells from different tissues were analyzed by RT-PCR and flow cytometry using also NHDFs. The presence of ET receptors in HDFs and SSc HDFs was performed on these cells by flow cytometry and western blot analysis. We found that all the fibroblasts tested express both ET_A and ET_B (Figure 10). We did not perform this analysis on HPAECs since it is well known from the literature only the presence of the ET_B receptor [34, 35].

4.2 MCR and CYP11B2

The presence of MCR mRNA was detected in HPFs, NHDFs and HPAECs by means of RT-PCR. HPFs and HPAECs showed the presence of mRNA for this receptor while it seems not to be present in NHDFs. After isolation of HDFs and SSc HDFs, western blot analyses were carried out on HPF, HDFs, SSc HDFs and HDAECs. The western blot analyses showed the presence of MCR in all the cell types analyzed with a difference of expression between HDFs and SSc HDFs (Figure 11).

To better understand the aldosterone biogenesis pathway, the presence of CYP11B2 was evaluated in HPF, HDFs, SSc HDFs and HDAECs by western blot analysis. All these cells showed the presence of this enzyme in equal amount (Figure 11B).

4.3 ET-1 effects on MCR and α-SMA

The presence of MCR and α-SMA mRNA was evaluated by RT-PCR in HPFs, NHDFs and HPAECs stimulated by ET-1 for 24 hours (Figure 12). The mRNA amounts do not change before and after stimulation with ET-1.

4.4 Activation of fibroblasts

In order to evaluate the activation of fibroblasts, protein levels of PDGF, collagen-1 and TGF-β were tested in the supernatants of HPF and NHDF cultures before and after stimulation with ET-1 for 24 or 48 hours using commercial ELISA kits (Figure 13). The data obtained are reported in Table 4 and the statistical analysis shows significant differences in: HPFs collagen-1 production only after 24 hours of incubation with ET-1 ($p<0.0001$); NHDFs collagen-1 production after 24 and 48 hours ($p<0.0001$ and $p<0.0002$ respectively); HPFs TGF-β production after 24 and 48 hours ($p<0.0006$ and $p<0.0013$ respectively); NHDFs TGF-β production after 24 and 48 hours ($p<0.0001$ and $p<0.0392$ respectively); PDGF production in both HPFs and NHDFs only after 24 hours of incubation ($p<0.0001$).

4.5 Oxidative stress

The production of ROS was analyzed with CM-H_2DCFDA, a chloromethyl derivative of H_2DCFDA that passively diffuses into cells, where its acetate groups are cleaved by intracellular esterases and its thiol-reactive chloromethyl group reacts with intracellular glutathione and other thiols; subsequent oxidation yields a fluorescent adduct that is trapped inside the cells. In HPFs, SSc HDFs and HPAECs, ET-1 stimulated the production of ROS only after 24-hour incubation and this production was inhibited by the use of the ET_B blocker BQ788. In NHDFs and HDFs there was absence of ROS production both after short and long stimulation with ET-1 (Figure 14). In HPFs, SSc HDFs and HPAECs, but not in NHDFs and HDFs, also aldosterone stimulated the

production of ROS after a short incubation of 40 minutes and this production was inhibited by pre-incubation with spironolactone (Figure 15). Same results were found in HPFs, SSc HDFs and HPAECs stimulated by ET-1 for 24 hours with or without spironolactone (Figure 16).

5. DISCUSSION AND CONCLUSIONS

Since its discovery, ET-1 is known to be a vasoconstrictor mediator but nowadays other different effects have been identified; indeed the action of this peptide is involved in cell adhesion, inflammation and angiogenesis. All these aspects have a pivotal role in the pathogenesis of SSc but also of PAH: high levels of this peptide are present in the plasma, derma and internal organs of these patients [69-72]. The recent literature [35, 58] reports apparently contradictory actions of ET-1 on pulmonary ECs *in vitro*, while on SMCs it stimulates muscle contraction by binding to ET_A; indeed the interaction with fibroblast stimulates fibrogenesis increasing production of extracellular matrix proteins. It is known that the ET-1 bound to ET_B expressed on ECs initiates a signal cascade that increases NO production by eNOS activation. This fact is in contrast with the observation that ET-1 may represent a stimulus for the endothelial aldosterone synthesis through PPAR coactivator-1α, steroidogenesis factor-1 and CYP11B2. Therefore the produced aldosterone is able to block ET_B pathway. Indeed in PAH patients low levels of NO are found and for this reason therapeutic agents mimicking the NO action have been introduced into the PAH treatment.

The starting point of this study was the evaluation of the presence of ET-1 receptors on analyzed cells. At the beginning, their expression was tested with flow cytometry and RT-PCR in HPFs and NHDFs to find possible differences in fibroblasts derived from different tissues. Subsequently, this analysis was extended also to dermal fibroblasts from skin biopsies performing flow cytometry and western blot analyses. The evaluation of the presence of these receptors was relevant for the following experiments regarding the effects of ET-1. The detection of the mediators and components of fibrosis (TGF-β, PDGF and collagen-1) production in the supernatant of dermal and lung fibroblasts revealed that these molecules are generally increased after stimulation with ET-1. The high production of collagen-1 indicates the ability of ET-1 to promote fibrosis in various tissues as occurs in SSc. Therefore the endothelinic stimulus increases synthesis of the fundamental component of the extracellular matrix and the

production of profibrotic molecules. The potential of dermal and lung fibroblasts to differentiate into myofibroblats after stimulation with ET-1 was evaluated by RT-PCR of mRNA encoding for α-SMA. Transdifferentiation is a very important event in the pathogenesis of PAH and other fibrosing diseases. In our experiments, ET-1 did not change the transcription of α-SMA mRNA; our hypothesis is that cells need a further stimulus to start the transdifferentiation process. To investigate the role of aldosterone in the pathogenesis of PAH, the presence of the mRNA encoding for MCR was evaluated by RT-PCR in HPFs, NHDFs and HPAECs before and after 24-hour stimulation with ET-1: the amount of mRNA does not change. The presence of MCR and CYP11B2 was analyzed using western blot analysis in HPFs, HDFs, SSc HDFs and HPAECs; MCR was detected in different amount in the various cells analyzed whereas CYP11B2 did not behave in the same way; MCR is present in low concentration in HDFs and MCR mRNA was not detectable in NHDFs. All these data suggest that fibroblasts behavior from different body districts may be different. Through flow cytometry, we were able to prove that a 24-hour stimulation with ET-1 increases ROS production. This datum confirms that oxidative stress mediated by ET-1 is involved in the pathogenesis of PAH at HPAECs level. The block of ET_B through a specific receptor blocker (BQ788) inhibited the synthesis of ROS in these cells that displayed an amount of ROS production similar to unstimulated cells. Analogous results were obtained with similar stimulation experiments conducted with ET-1 on HPFs that express both ET_A and ET_B receptors. Also in this case the block of ET_B led to lower production of basal ROS; on the contrary the ET_A block did not involve any effect on the synthesis of ROS after ET-1 stimulation. This results suggested that the ET-1/ET_B interaction is important for the oxidative stress in both HPAECs and HPFs. Since the induction of oxidative stress needs an incubation of 24 hours with ET-1 it is possible that this incubation is necessary for the activation of signaling cascade for the synthesis of aldosterone. A very interesting thing is the different behavior of dermal fibroblasts derived from healthy donors and SSc patients. Both kinds of fibroblasts express ET_A and ET_B but only SSc HDFs showed a ROS production as HPAECs and HPFs. We have incubated cells with aldosterone for 40 minutes and this short incubation was

sufficient to induce ROS production in HPAECs, HPFs and SSc HDFs. The possible correlation between ET-1 and aldosterone is supported by the absence of ROS production when cells were incubated with spironolactone, the aldosterone inhibitor, and ET-1 for 24 hours.

These results suggest that ET-1 is an important molecule involved in pathogenesis of PAH for its ability to activate fibrosis but also for its indirect role in oxidative stress inducing the production of aldosterone that seems to be the true responsible for ROS production.

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TABLES AND FIGURES

Table 1. Hemodynamic definition of pulmonary hypertension.

Definition	Characteristics	Clinical group(s)
PH	PAPm ≥25 mmHg	All
Pre-capillary PH	PAPm ≥25 mmHg PAWP ≤15 mmHg	1. PAH 3. PH due to lung diseases 4. Chronic thromboembolic PH 5. PH with unclear and/or multifactorial mechanisms
Post-capillary PH	PAPm ≥25 mmHg PAWP >15 mmHg	2. PH due to left heart disease 5. PH with unclear and/or multifactorial mechanisms
Isolate post-capillary PH	DPG <7 mmHg and/or PVR ≤3 WU	
Combined post-capillary and pre-capillary PH	DPG ≥7 mmHg and/or PVR >3 WU	

Table 2. Clinical classification of pulmonary hypertension (Nice, 2013).

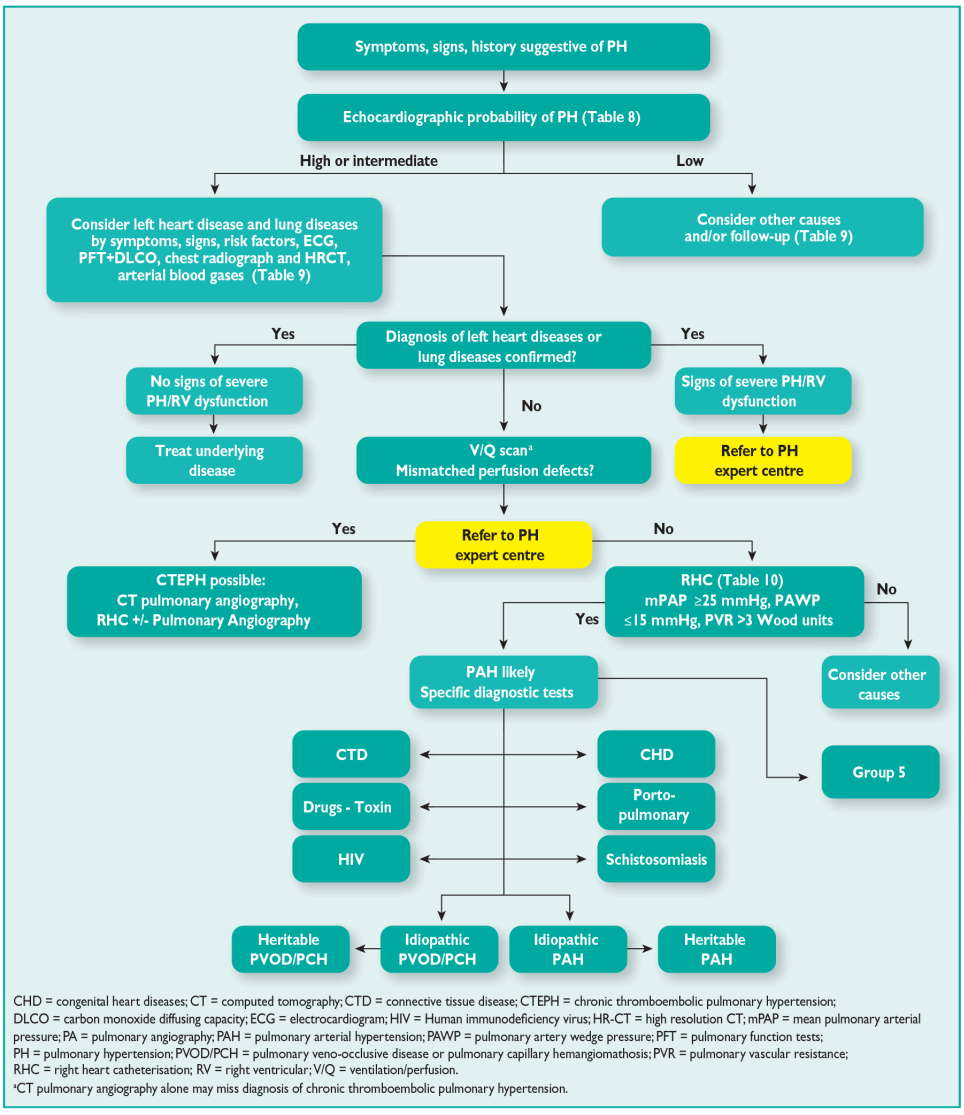
1. PAH
1.1 Idiopathic
1.2 Heritable
1.2.1 BMPR2 mutation
1.2.2 Other mutations
1.3 Drug and toxin induced
1.4 Associated with:
1.4.1 Connective tissue disease
1.4.2 HIV infection
1.4.3 Portal hypertension
1.4.4 Congenital heart disease
1.4.5 Schistosomiasis
1'. Pulmonary veno-occlusive disease and/or pulmonary capillary hemangiomatosis
1'.1 Idiopathic
1'.2 Heritable
1'.2.1 EIF2AK4 mutation
1'.2.2 Other mutations
1'.3 Drug, toxin and radiation induced
1'.4 Associated with:
1'.4.1 Connective tissue disease
1'.4.2 HIV infection
1''. Persistent PH of the newborn
2. PH due to left heart disease
2.1 Left ventricular systolic dysfunction
2.2 Left ventricular diastolic dysfunction
2.3 Vascular disease
2.4 Congenital/acquired left heart inflow/outflow tract
2.5 Congenital/acquired pulmonary veins stenosis
3. PH due to lung disease and/or hypoxia
3.1 Chronic obstructive pulmonary disease
3.2 Interstitial lung disease
3.3 Other pulmonary disease with mixed respective and obstructive pattern
3.4 Sleep-disordered breathing
3.5 Alveolar hypoventilation disorders
3.6 Chronic exposure to high altitude
3.7 Developmental lung disease
4. Chronic thromboembolic PH and other pulmonary artery obstructions
4.1 Chronic thromboembolic PH
4.2 Other pulmonary artery obstructions
4.2.1 Angiosarcoma
4.2.2 Others intravascular tumors
4.2.3 Arteritis
4.2.4 Congenital pulmonary arteries stenoses
4.2.5 Parasites
5. PH with unclear and/or multifactorial mechanisms
5.1 Hematological disorders
5.2 Systemic disorders, sarcoidosis, pulmonary histiocytosis, lymphangioleiomyomatosis
5.3 Metabolic disorders
5.4 Others

Table 3. Cellular source of ET-1, ET-2 and ET-3.

ET-1	ET-2	ET-3
ECs	Kidney epithelial cells	Neuronal stromal cells
Cardiomyocytes	Cardiomyocytes	Glial cells
Aortic vascular SMCs	Throphoblastic cells	Adrenal cells
Kidney epithelial cells	Uterine glandular epithelial cells	Intestinal epithelial cells
Neurosecretory nerve endings in neurohypophysis	Gastroentestinal stromal cells	Kidney epithelial cells
Astrocytes		Lung epithelial cells
Kidney mesangial cells		
Sertoli cells		
Endometrial cells		
Breast epithelial cells		
Leukocytes		
Myofibroblasts		
Bile ductus epithelial cells		
Neuronal stromal cells		

Table 4. Collagen-1, TGF-β and PDGF amount in cell culture supernatants.

HPFs	CTRL 24h	ET-1 24h	CTRL 48h	ET-1 48h
Col-1 (ng/mL)	6.645±0.088	7.533±0.126	6.670±0.052	7.900±0.330
TGF-β (pg/mL)	1190.047±5.263	1252.193±1.055	1276.790±0.561	1242.780±32.087
PDGF (pg/mL)	22.550±0.362	24.215±0.066	21.058±0.413	21.068±0.413
NHDFs	**CTRL 24h**	**ET-1 24h**	**CTRL 48h**	**ET-1 48h**
Col-1 (ng/mL)	6.855±0.150	7.747±0.051	6.887±0.059	6.865±0.058
TGF-β (pg/mL)	627.44±18.257	679.852±0.863	641.695±98.339	887.110±9.653
PDGF (pg/mL)	20.675±0.050	22.877±0.491	24.647±0.412	24.505±0.243



CHD = congenital heart diseases; CT = computed tomography; CTD = connective tissue disease; CTEPH = chronic thromboembolic pulmonary hypertension; DLCO = carbon monoxide diffusing capacity; ECG = electrocardiogram; HIV = Human immunodeficiency virus; HR-CT = high resolution CT; mPAP = mean pulmonary arterial pressure; PA = pulmonary angiography; PAH = pulmonary arterial hypertension; PAWP = pulmonary artery wedge pressure; PFT = pulmonary function tests; PH = pulmonary hypertension; PVOD/PCH = pulmonary veno-occlusive disease or pulmonary capillary hemangiomathosis; PVR = pulmonary vascular resistance; RHC = right heart catheterisation; RV = right ventricular; V/Q = ventilation/perfusion.

[a]CT pulmonary angiography alone may miss diagnosis of chronic thromboembolic pulmonary hypertension.

Figure 1. Diagnostic algorithm of PH [6].

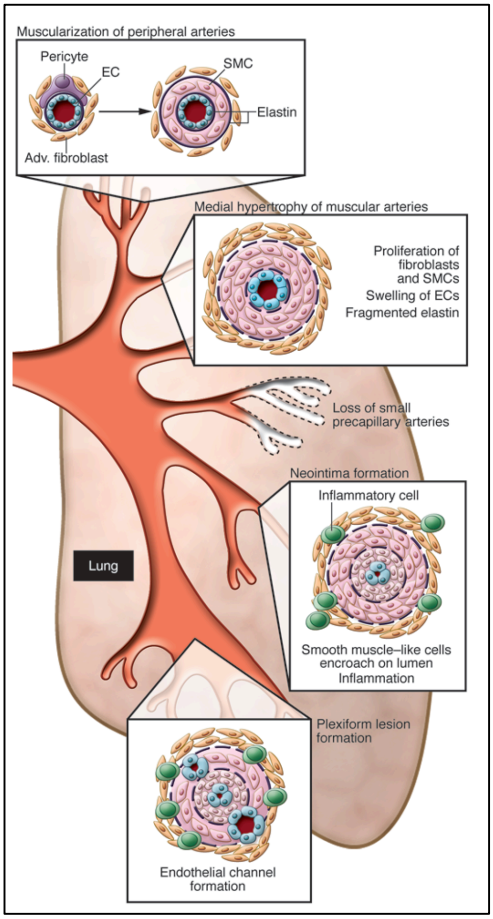


Figure 2. Vascular abnormalities associated with PAH [13].

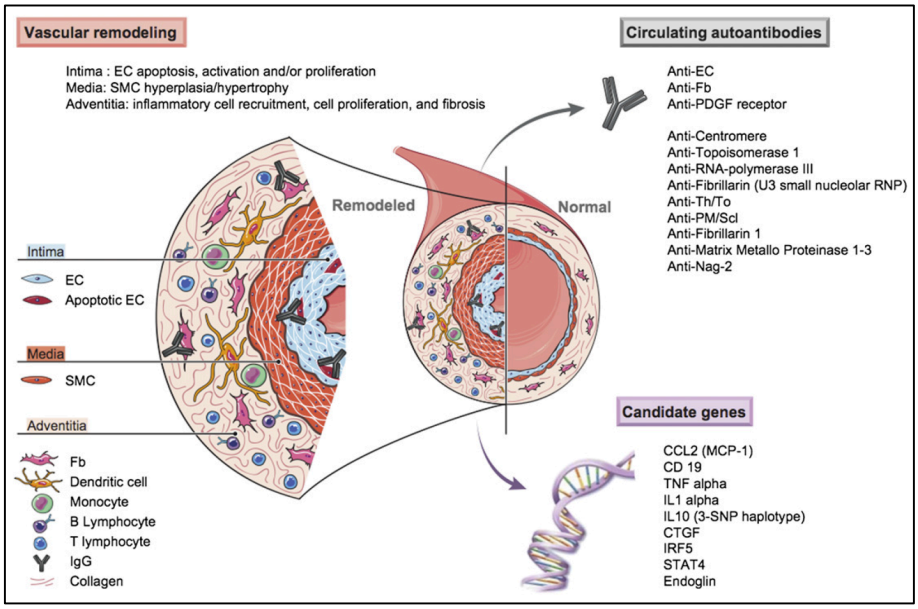


Figure 3. Pulmonary vascular remodeling in PAH [16].

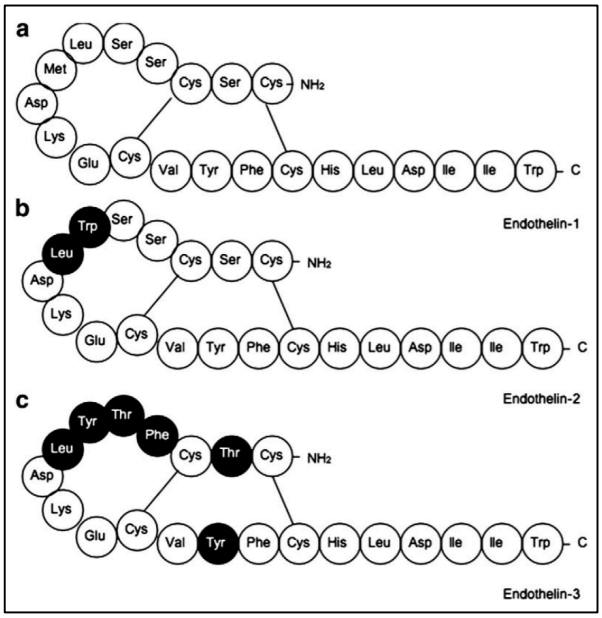


Figure 4. Endothelin structure [34].

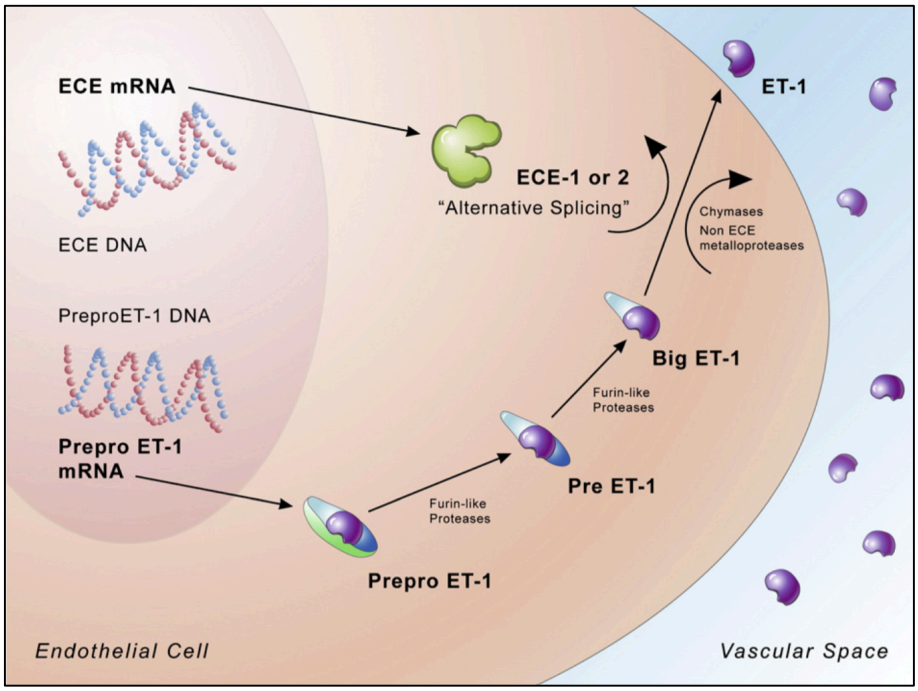


Figure 5. ET-1 biosynthetic pathway [34].

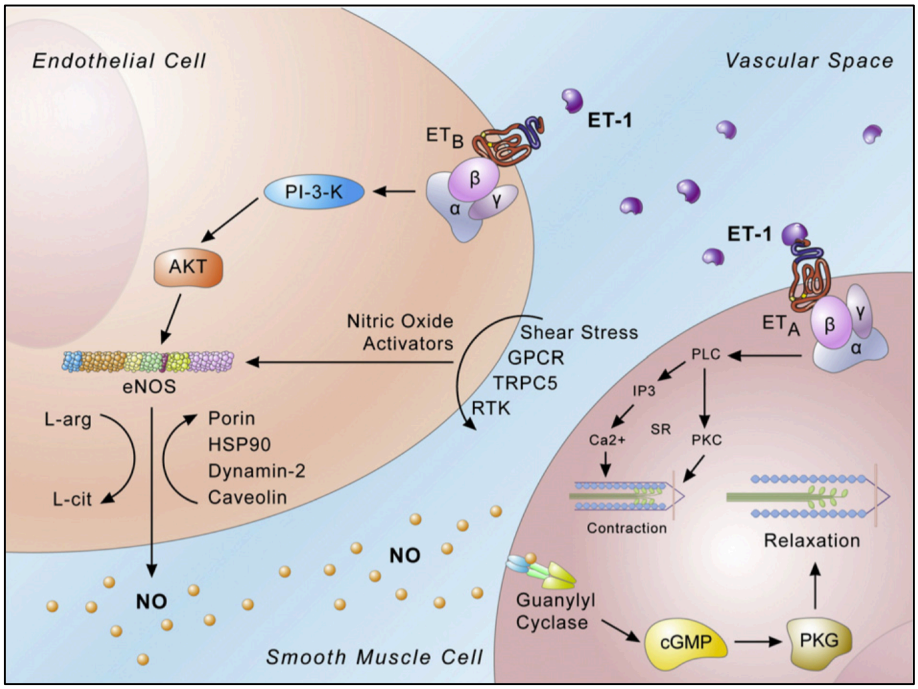


Figure 6. Endothelin signaling in vascular space [34].

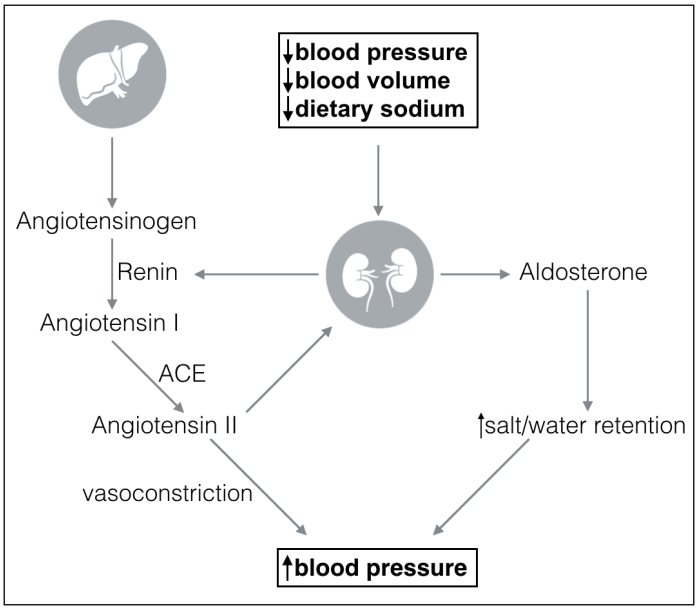


Figure 7. The renin-angiotensin-aldosterone system.

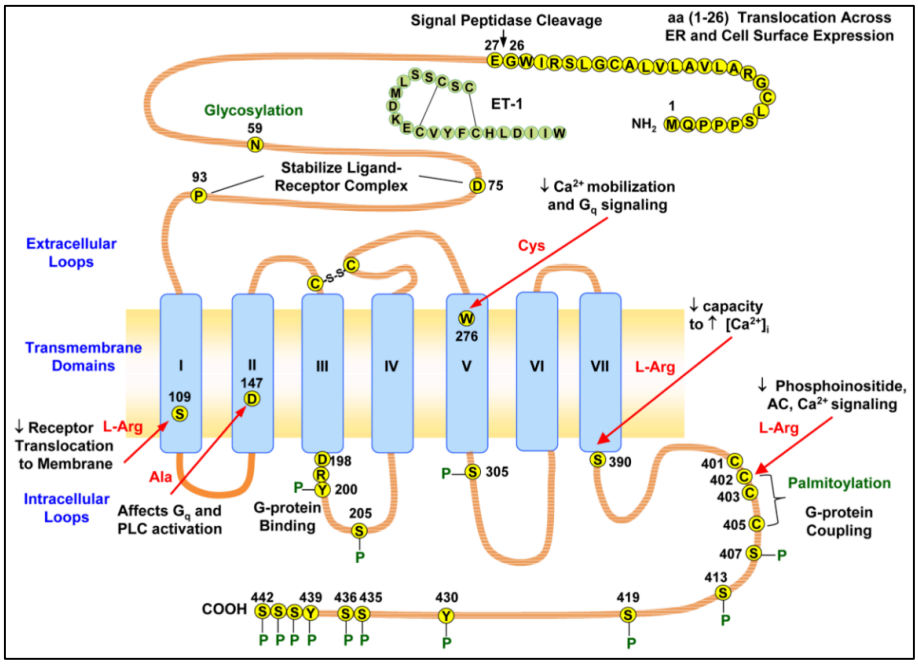


Figure 8. ET_B structure [67].

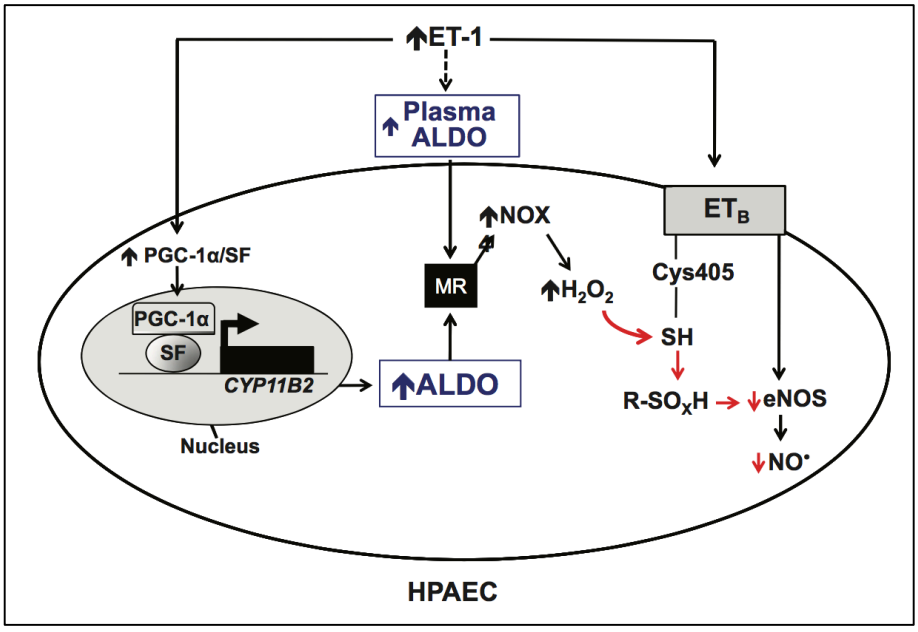


Figure 9. A proposed mechanism by which hyper-aldosteronism decreases NO generation and eNOS activation in PAH [35].

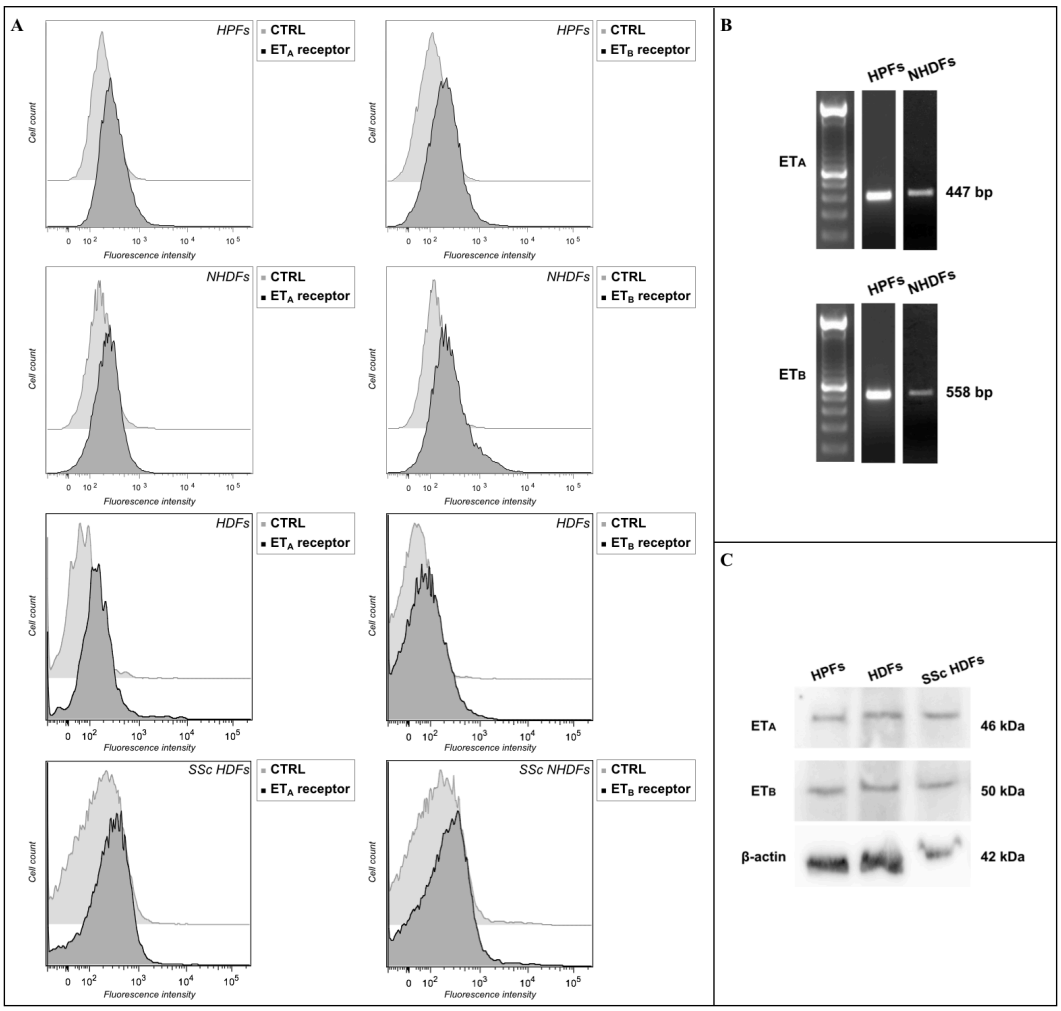


Figure 10. ET receptors analyses in fibroblasts. Expression of ET receptors is analyzed with flow cytometry in HPFs, NHDFs, HDFs and SSc HDFs (A), RT-PCR in HPFs and NHDFs (B), western blot analysis in HPFs, HDFs and SSc HDFs (C). Every kind of analysis shows the presence of both ET receptors in the cells analyzed.

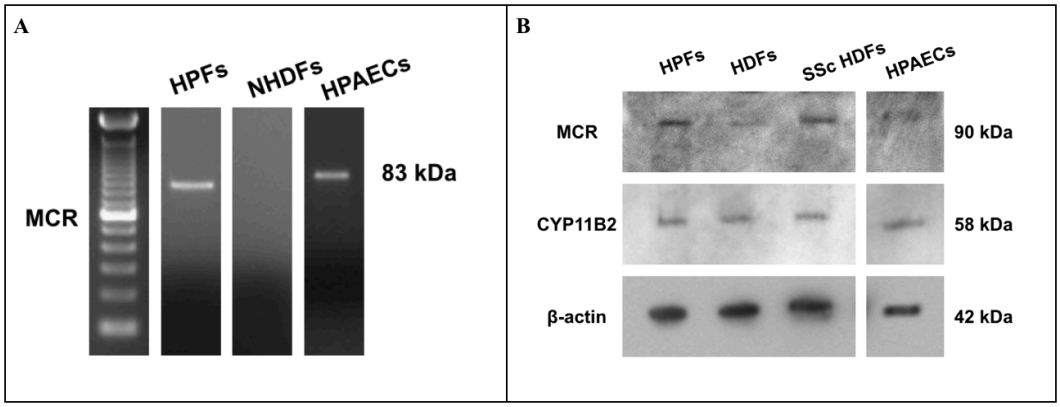


Figure 11. MCR and CYP11B2 expression in fibroblasts. (A) RT-PCR shows the presence of MCR mRNA in HPFs and HPAECs but not in NHDFs. (B) Western blot analysis confirms the presence of MCR in HPFs and HPAECs and shows a difference receptor amount in cells analyzed: SSc HDFs express MCR 1.27 times more than HPFs and 1.32 times more than HDFs; HPFs and HDFs show a difference of MCR expression between them of 1.03 times; HPAECs express MCR 1.48, 1.54, 1.16 times more than HPFs, HDFs and SSc HDFs respectively. CYP11B2 amount is similar in every kind of analyzed fibroblasts but is 1.40 times more expressed in HPAECs.

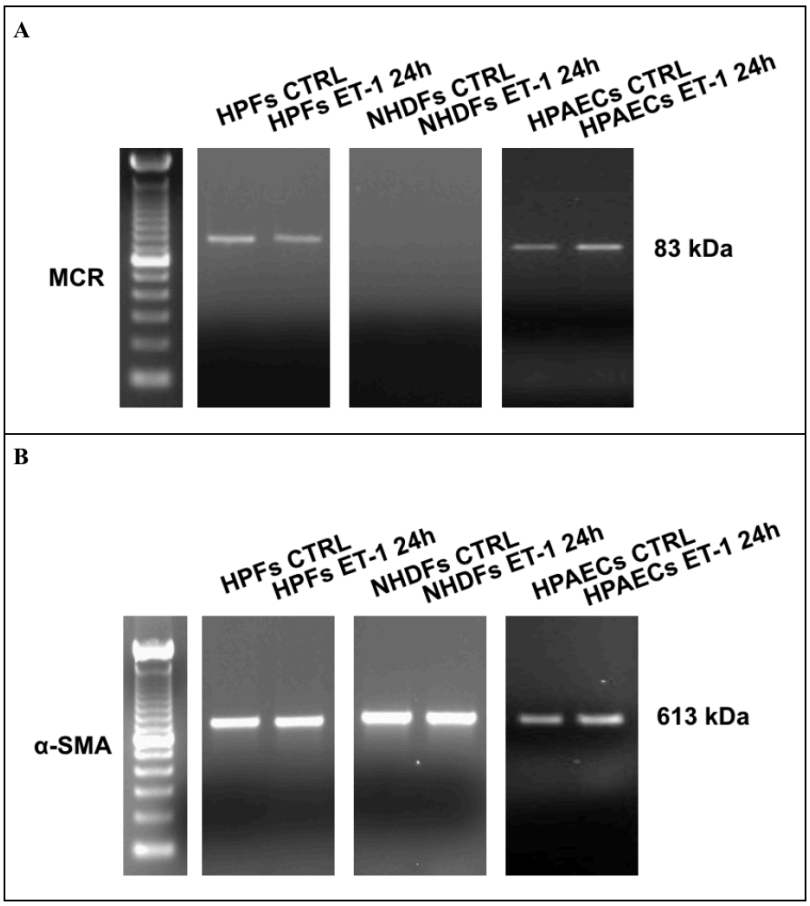


Figure 12. ET-1 effects on MCR (A) and α-SMA (B) on HPFs, NHDFs and HPAECs. RT-PCR does not show differences in mRNA amount of MCR or α-SMA in cells analyzed after ET-1 stimulation for 24hs.

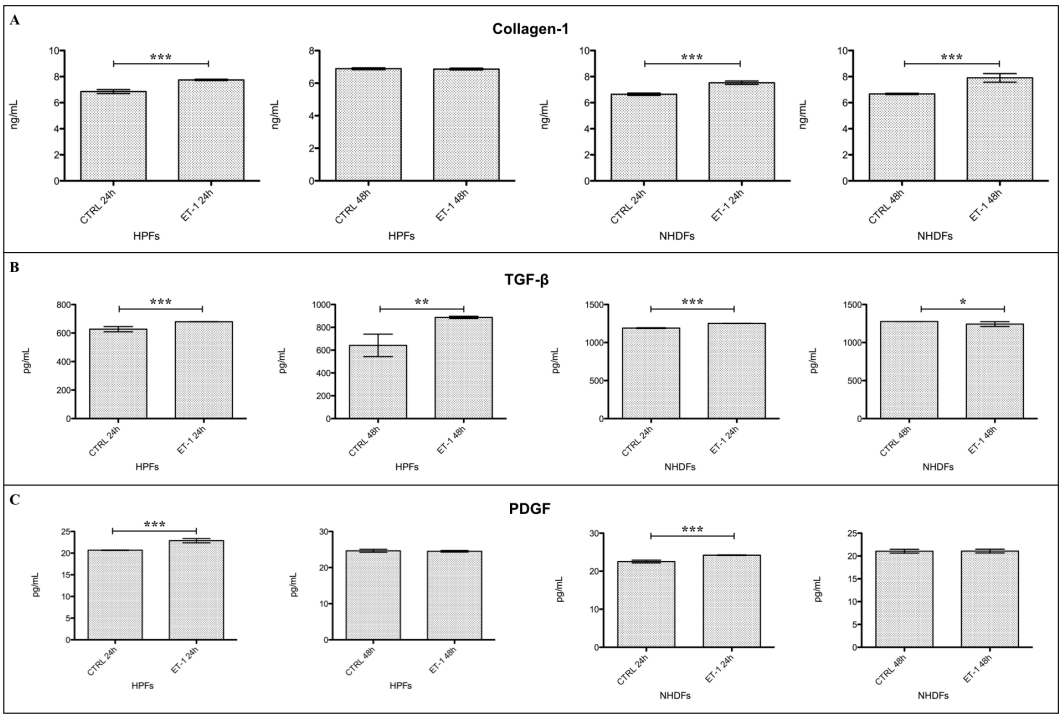


Figure 13. Fibroblasts activation after ET-1 stimulation. Activation of fibroblast is analyzed measuring levels of collagen-1 (A), TGF-β (B) and PDGF (C) in supernatants of HPFs and NHDFs after 24 or 48 hours of incubation with ET-1. A stimulation of 24 hours is sufficient to induce the production of collagen-1, TGF-β and PDGF in HPFs and NHDFs. After 48 hours TGF-β is released by HPFs while in NHDF supernatants collagen-1 increases together with TGF-β. (*** $p<0.0001$; ** $p<0.001$; * $p<0.05$)

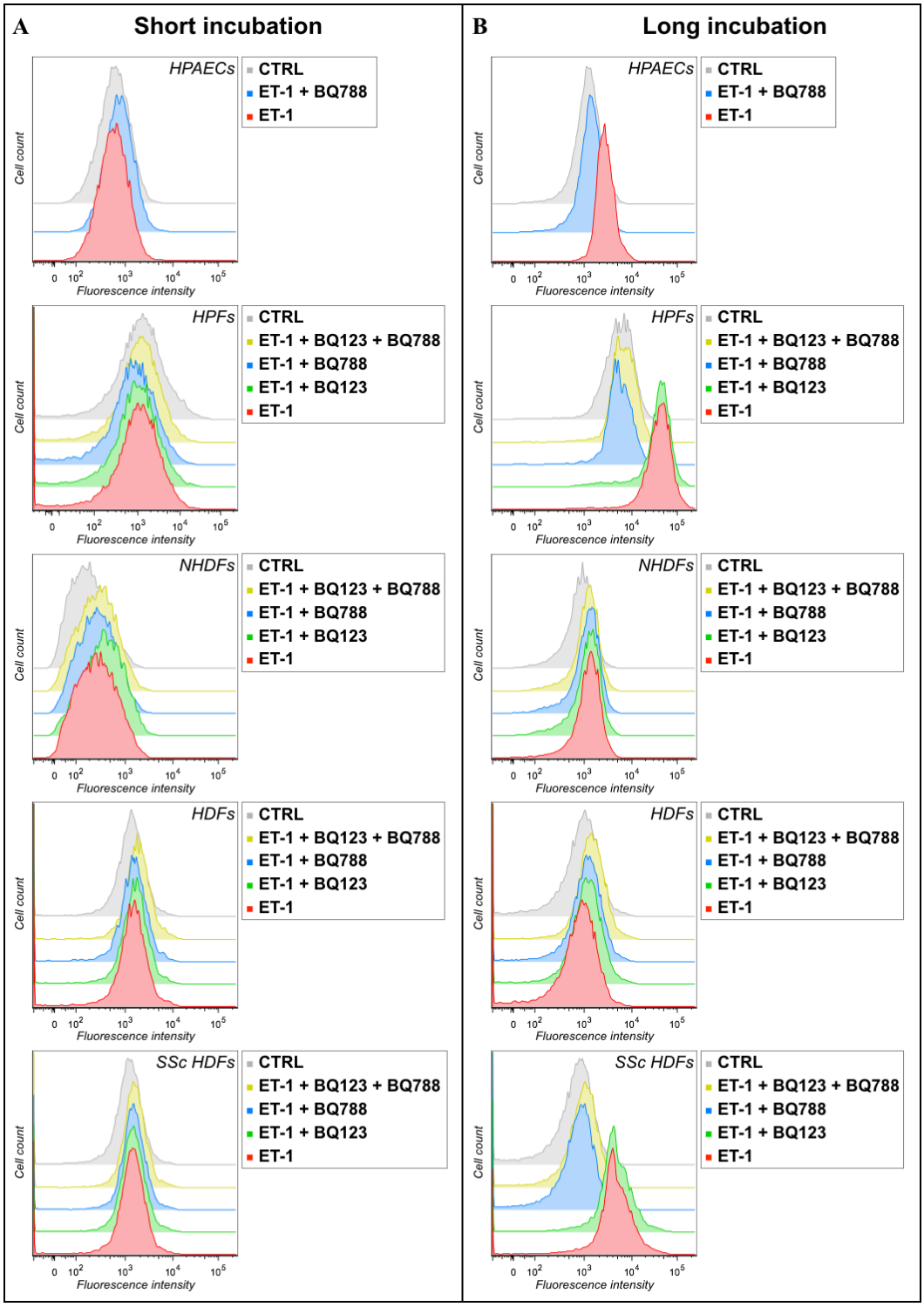


Figure 14. Evaluation of oxidative stress after 40 minutes (A) or 24 hours (B) of ET-1 stimulation. ET-1 stimulates ROS production only after the 24-hour incubation in HPFs, SSc HDFs and HPAECs; this production is inhibited by blocking ET_B receptor using BQ788. In NHDFs and HDFs there is absence of oxidative stress formation after both short and long ET-1 stimulation.

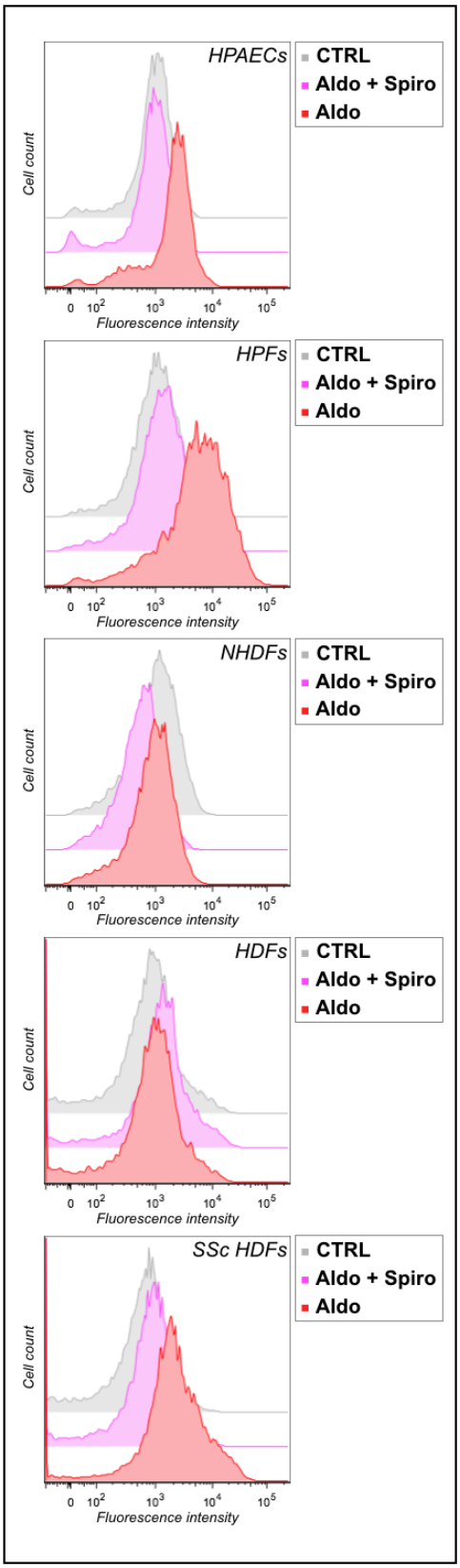


Figure 15. Evaluation of oxidative stress after 40 minutes of aldosterone stimulation. Aldosterone stimulates the production of ROS after a short incubation in HPFs, SSc HDFs and HPAECs, but not in NHDFs and HDFs; this production is inhibited by pre-incubation with spironolactone.

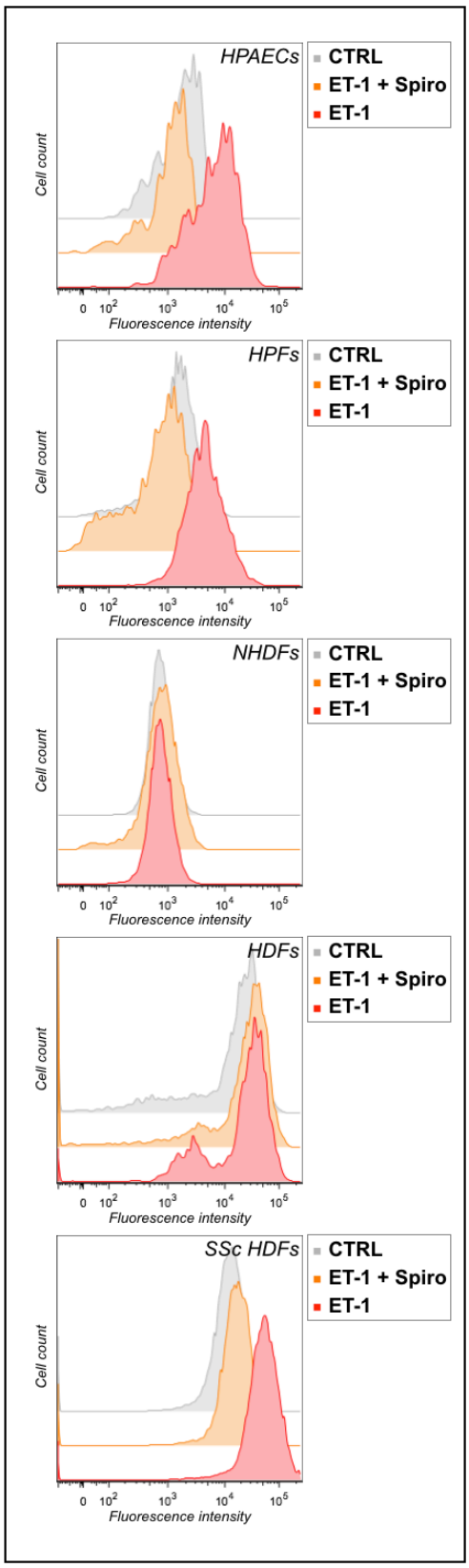


Figure 16. Evaluation of oxidative stress after 24 hours of ET-1 stimulation with or without spironolactone co-incubation. ET-1 stimulates the production of ROS after a long incubation in HPFs, SSc HDFs and HPAECs, but not in NHDFs and HDFs; this production is inhibited by co-incubation with spironolactone.

RESEARCH PRODUCTS

- A Barbieri, M Dolcino, E Tinazzi, A Rigo, G Argentino, G Patuzzo, A Ottria, R Beri, A Puccetti, C Lunardi. *Characterization of CD30/CD30L + Cells in Peripheral Blood and Synovial Fluid of Patients with Rheumatoid Arthritis.* Journal of Immunology Research 01/2015; 2015:1-10. DOI:10.1155/2015/729654
- M Dolcino, A Ottria, A Barbieri, G Patuzzo, E Tinazzi, G Argentino, R Beri, C Lunardi, A Puccetti. *Gene Expression Profiling in Peripheral Blood Cells and Synovial Membranes of Patients with Psoriatic Arthritis.* PLoS ONE 01/2015; 10(6):e0128262. DOI:10.1371/journal.pone.0128262
- E Tinazzi, A Puccetti, G Patuzzo, A Barbieri, G Argentino, F Confente, M Dolcino, R Beri, G Marchi, A Ottria, D Righetti, M Rampudda, C Lunardi. *Endothelin Receptors Expressed by Immune Cells Are Involved in Modulation of Inflammation and in Fibrosis: Relevance to the Pathogenesis of Systemic Sclerosis*. Journal of Immunology Research 01/2015; 2015:1-11. DOI:10.1155/2015/147616
- M Dolcino, G Patuzzo, A Barbieri, E Tinazzi, M Rizzi, R Beri, G Argentino, A Ottria, C Lunardi, A Puccetti. *Gene Expression Profiling in Peripheral Blood Mononuclear Cells of Patients with Common Variable Immunodeficiency: Modulation of Adaptive Immune Response following Intravenous Immunoglobulin Therapy*. PLoS ONE 05/2014; 9(5):e97571. DOI:10.1371/journal.pone.0097571
- E Tinazzi, A Barbieri, A Rigo, G Patuzzo, R Beri, R Gerli, G Argentino, A Puccetti, C Lunardi. *In rheumatoid arthritis soluble CD30 ligand is present at high levels and induces apoptosis of CD30(+)T cells*. Immunology letters 01/2014; DOI:10.1016/j.imlet.2014.01.007