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INVESTIGATION OF THE MOLECULAR MECHANISMS INDUCING VASCULAR DAMAGE IN SYSTEMIC SCLEROSIS

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TABLE OF CONTENTS

ATTESTATION OF AUTHORSHIP

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person except that which appears in the citations and acknowledgements. Nor does it contain material, which to a substantial extent I have submitted for the qualification for any other degree of another university or other institution of higher learning.

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ABBREVIATION

α-SMA	alpha-smooth muscle actin
4-HNE	4-hydroxynonenal, a common byproduct of lipid peroxidation during oxidative stress
ACR	American College of Rheumatology
ADAM17/NOTCH	Disintergrin and metalloproteinase domain-containing protein 17 involved in the activation of the Notch signaling pathway
AECA	Anti-endothelial cell antibodies
ALK5	Activin receptor-like kinase 5
c-Abl	c-Abl protein kinase
CTGF	Connective tissue growth factor
dcSSc	diffuse cutaneous systemic sclerosis
EMT	Epithelial-to-Mesenchymal Transition
EndMT	Endothelial-to-Mesenchymal Transition
ERK	Extracellular signal-regulated kinases
ET-1	Endothelial-1
EULAR	European League Against Rheumatism
FSP-1	Fibroblast specific protein-1
GSK-3β	Glycogen synthase kinase 3β
GTPase	Guanosine triphosphate intracellular signaling

HOCl	Hypochlorous acid
HPASMCs	Human pulmonary artery smooth muscle cells
HPMECs	Human pulmonary microvascular endothelial cells
I-EndMT	Induced EndMT
IL-1β	Interleukin-1β
JAMs	Junctional adhesion molecules
lcSSc	limited cutaneous systemic sclerosis
miRNAs	MicroRNAs
MMP	Matrix metalloproteinase-1
MVECs	Microvascular endothelial cells
NADPH oxidase (NOX)	Nicotinamide adenine dinucleotide phosphate oxidase
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
$O_2^{\cdot -}$	Superoxide radical
$^{\cdot}OH$	Hydroxyl radical
$ONOO^-$	Peroxynitrite
p38MAPK	P38 Mitogen-activated protein kinases
PAH	Pulmonary artery hypertension
PAI-1	Plasmin activator inhibitor 1
PDGF	Platelet derived growth factors
PDGFR	Platelet derived growth factor receptor

PECAM 1	Platelet endothelial cell adhesion molecule-1
PI3K	Phosphoinositide 3-kinase
PKC-δ	Protein kinase Cδ
PTU	Propylthiouracil
Ras	A small GTP-binding protein
ROS	Reactive oxygen species
SSc	Systemic sclerosis
TGF-β	Transforming growth factor-β
TIMPs	Tissue inhibitors of metalloproteinases
TNF-α	Tumor necrosis factor-alpha
VE-Cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
vWF	Von Willebrand factor

ABSTRACT

Purposes - This study was conducted to investigate whether oxidative stress and Endothelial-to-Mesenchymal Transition (EndMT) may be part of the molecular machinery inducing vascular damage in systemic sclerosis (SSc). The possibility that iloprost, a drug commonly used in SSc therapy, might modulate the above-mentioned biological phenomena was also investigated.

Results - Sera from SSc patients markedly increased ROS levels, proliferation, and collagen synthesis in human pulmonary microvascular endothelial cells (HPMECs). Interestingly, SSc sera taken after 5 hours of iloprost infusion could attenuate ROS levels and collagen synthesis.

Preliminary results show that SSc sera induced the conversion of ECs into myofibroblasts through decreasing the endothelial marker, von Willebrand Factor, and increasing α-smooth muscle actin, the myofibroblastic marker.

Conclusion - Exposition of HPMECs to pro-oxidant factors present in SSc sera increased disease-associated physio-pathological phenomena such as intracellular ROS levels and collagen synthesis. Reduction of the above-mentioned phenomena by iloprost suggests a potential antioxidant effect of this drug. Preliminary data demonstrate the presence of an SSc sera-induced EndMT, indicating this phenotypic shift as an important etiological mechanism of SSc-associated vascular damage and a potential therapeutic target to inhibit obliterative vascular disorder and tissue fibrosis.

1. INTRODUCTION

1.1. Systemic sclerosis

Systemic sclerosis (scleroderma, SSc) is a complex multisystem autoimmune disease of unknown etiology characterized by dysregulated immune system, progressive fibrosis of skin and visceral organs in which extensive vascular alterations are prominent and severe [41]. There are two major subgroups in the commonly accepted classification of systemic sclerosis: limited cutaneous systemic sclerosis (lcSSc) and diffuse cutaneous systemic sclerosis (dcSSc). The degree of skin fibrosis, immunological profile, and microvascular dysfunction determines this clinical classification of the disease [51]. In lcSSc, skin fibrosis is restricted to the fingers, distal extremities and face, whereas in dcSSc, the trunk and proximal extremities are also affected. In patients with lcSSc, Raynaud's phenomenon is present for several years before fibrosis appears, whereas patients with dcSSc have rapid disease progression with extensive skin changes and early development of visceral organ complications [2]. Autoantibodies are not only predictive value for clinical evaluation and prognosis but also signs to distinguish between lcSSc and dcSSc. lcSSc is commonly associated with centromere-specific antibodies which occur in 50 to 90% of patients, whereas dcSSc is more often associated with topoisomerase I-or RNA polymerase III-specific antibodies [52, 112]. This classification can be useful, but none of the proposed classifications sufficiently reflects the heterogeneity of the clinical manifestations of systemic sclerosis. Some individuals present with hallmark clinical and serological features of systemic sclerosis in the absence of detectable skin involvement; others manifest the overlap of systemic sclerosis with another connective tissue disease, such as lupus erythematosus, rheumatoid arthritis, polymyositis .

Systemic sclerosis can cause severe dysfunction and failure of almost any internal organ. Major organ involvement leads to decreased survival in SSc. Pulmonary fibrosis and pulmonary arterial hypertension (PAH) cause more than half of all SSc-related deaths. In addition, severe complications of the kidneys, heart, lungs, and gastrointestinal tract generally develop within 3 years of disease onset, particularly in patients with dcSSc [85].

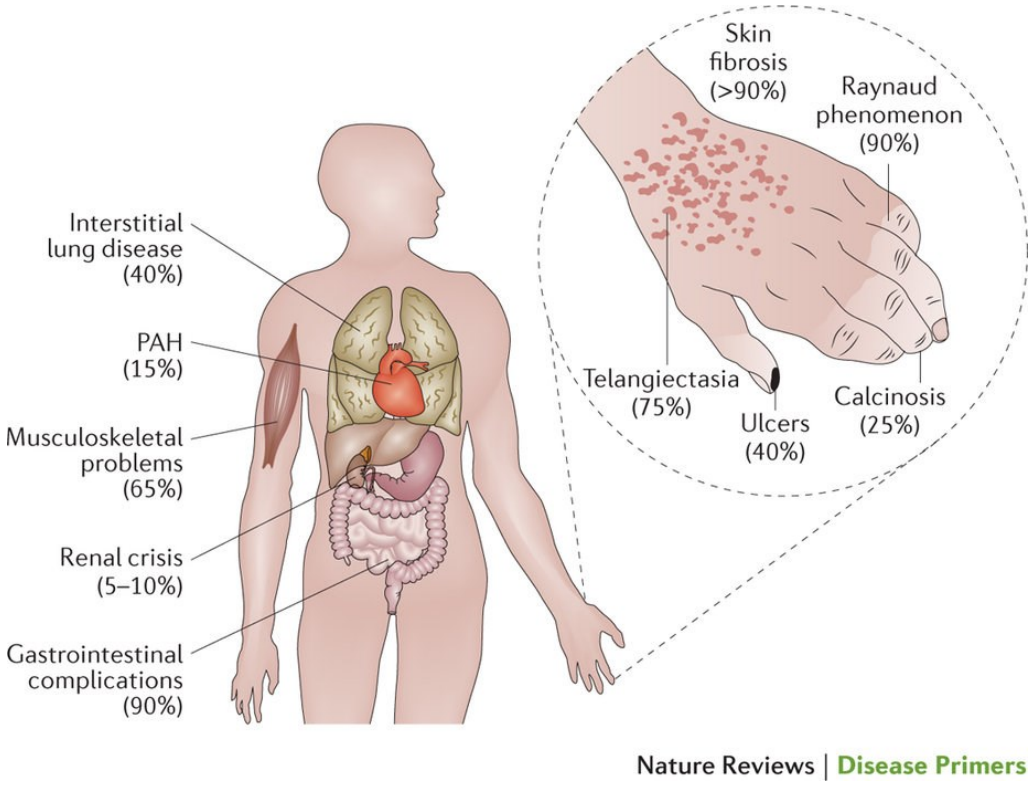


Figure 1.1. Organ complications associated with systemic sclerosis. The uncontrolled fibrosis and scaring of the skin and internal organs in systemic sclerosis leads to severe and sometimes life-threatening complications. The average frequency of the specific complications is indicated in parentheses. PAH, pulmonary arterial hypertension [2].

1.1.1. Epidemiology

The results of studies of prevalence and incidence estimates of SSc show a substantial variation across geographic regions. Prevalence and incidence of SSc appears to be greater in populations of European ancestry and lower in

Asian groups. Lower estimates of prevalence (<150 per million) and incidence (<10 per million per year) have been recorded in northern Europe and Japan, whereas higher estimates of prevalence (276-443 per million) and incidence (14-21 per million per year) have been reported in southern Europe, North America and Australia [7]. The 2013 revised classification criteria - the American College of Rheumatology (ACR)-European League Against Rheumatism (EULAR) criteria include patients with centromere-specific antibodies and limited cutaneous involvement. As a result, the estimated prevalence of SSc based on the ACR-EULAR classification criteria was much higher than previously published estimates [59].

Like other connective tissue diseases, SSc is sex dependent and occurs more common in women. Female/male ratio ranging from 3:1 to 14:1 and it occurs more frequently in the fourth or fifth decades of life. However, male sex has been consistently shown to be a poor prognostic factor in SSc. In addition, race and ethnic groups are related to distinct phenotypic profiles, and there is a trend towards less favorable outcomes in Africa American patients.

1.1.2. Endothelial Dysfunction in Systemic sclerosis

In SSc, the vasculopathy is one of the earliest pathological events, characterized by endothelial cell activation, and altered vascular tone. The fact that the vascular manifestations consistently precede tissue fibrosis suggests that microvascular endothelial cells (MVECs) are the primary target in this disease. The pathological alterations are accompanied by the presence of proinflammatory cytokines and angiogenic regulatory factors, and the loss of redox control. This complex interaction involves in a number of cells types, particularly the endothelial cells. The vascular endothelium provides a compatible interface to facilitate blood circulation as well as a multifunctional

interface between blood and all internal organs [1]. The vascular endothelium participates in regulating coagulation and fibrinolysis, permeability, vasomotion, and inflammation. The term endothelial dysfunction is most often used to symbolize impairment of endothelium-dependent vasodilation; however, a more expanded definition also includes endothelial interactions with leukocytes, platelets, and regulatory substances [3].

The most prominent clinical vascular dysfunction in SSc is linked to dysregulation of vascular tone leading to vascular spasm and reduced blood flow. An imbalance between the levels of vasoconstrictor and vasodilator mediators is attributed for endothelial dysfunction in SSc. Among these vasodilator agents, nitric oxide (NO), produced by endothelial cells (ECs) is one of the most potent vasodilator. In SSc, the defects in NO production by endothelial nitric oxide synthetase reduce microvascular endothelial cells (MVECs) survival and promote apoptosis. Moreover, endothelin-1 (ET-1), a potent vasoconstrictor peptide originally isolated from ECs is upregulated in SSc sera and tissues. The imbalance between vasodilator and vasoconstrictor signals leaves MVECs vulnerable to apoptosis and promotes an environment of ischemia, hypoxia, and profibrosis.

Hypoxia is a possible trigger that mediates MVECs lesions. Hypoxia followed by reperfusion induces an inflammatory process and oxidative stress that leads to cellular injury. Human MVECs exposing to intermittent hypoxia induced the dysfunction of MVECs, associated with an increase in oxidative stress. Intriguingly, using antioxidants prevents the effect of hypoxia on MVECs.

It is widely accepted that there are defects of angiogenesis and vasculogenesis in SSc. Angiogenesis and vasculogenesis are processes in which new vessels

are formed from preexisting vessels and de novo vessels, respectively. These defects indicate defective role of MVECs differentiation in two processes. Of interest, vascular endothelial growth factor (VEGF) is a very potent angiogenic factor that is overexpressed in skin and sera of SSc patients, but there is evidence suggesting that VEGF receptor may be impaired in the cells based on the observation that MVECs isolated from SSc patients show reduced response to VEGF *in vitro*. In addition, failed release of bone marrow-derived progenitor cells, which have the potential to initiate vascular repair has been identified in SSc patients. The failure to compensate for vascular injury eventually leads to accumulative loss of capillaries and arterioles, resulting in the well-known vascular complications: painful ulcers on the digits, scleroderma renal crisis occurs in the kidneys, and PAH occurs in the lungs [81].

There appears to be a complex cross talk between MVECs, fibroblasts, and leukocytes, especially in early phases of SSc. It is suggested that junctional adhesion molecules (JAMs) are important elements in this interaction. The levels of JAM-A are unregulated in MVECs especially in early stage SSc, and the soluble JAM-A and JAM-C levels are higher in early stage SSc [80]. This underlines the role of JAMs in activating MVECs, particularly in early stage SSc in which there are more inflammatory infiltrates around the blood vessels.

Finally yet importantly, MVECs may activate fibroblasts and the immune system. As a result, activated fibroblasts precede the overproduction of collagen and other compartments of extracellular matrix (ECM).

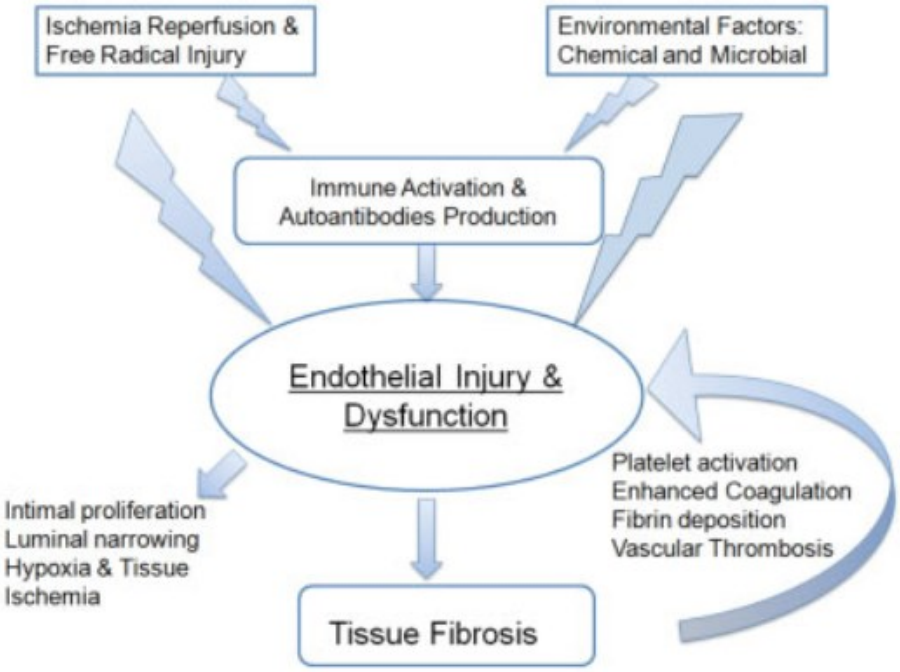


Figure 1.2. Pathogenesis of SSc vasculopathy. Endothelial injury and dysfunction are initiated by the actions of free radicals or chemical and microbial agents that injure the endothelium, either directly or indirectly. This injury is also initiated by the induction of immune activation and the generation of autoantibodies and activated cellular immunity. The vascular injury activated platelet and coagulation pathways, which results in vascular microthrombosis. The resulting vasculopathy is associated with intimal hyperplasia in the small arterioles, and the ensuing luminal narrowing results in tissue hypoxia and chronic ischemia. Released vascular products, in association with hypoxia and ischemia, collectively contribute to the activation of resident fibroblasts, which in turn perpetuates the vasculopathy by triggering vascular wall fibrosis [81].

1.1.3. Raynaud's Phenomenon

Raynaud's Phenomenon (RP) is the clinical manifestation of cutaneous vessel dysfunction involved thermal regulation of blood flow [124]. This exaggerated respond of the cutaneous circulation to cold exposure results in vasospasm and the characteristic pallor of the skin [39]. Its classical presentation involves the fingers turning white (ischemia), then blue (cyanosis), and then red (reperfusion) [56]. RP presents in almost SSc

patients, it is usually the first symptom of disease, and it begins before the onset of clinical signs of tissue fibrosis. RP occurs when the delicate balance between vasodilation and vasoconstriction is disturbed, in favor of vasoconstriction. The presence of RP and dysregulation of cutaneous vascular tone is a predictor of developing SSc. The locations of the most severe skin fibrosis follow the same distribution patterns as typical cutaneous body sites involved in thermoregulation (e.g., the fingers, feet, face, and lower arms), suggesting some causative relationship between the vascular disease and skin fibrosis.

Most research into the changes in vascular function in RP has so far focused on the endothelium. It has been suggested that RP in SSc patients may be triggered by endothelial injury [67] (Figure 1.2). Endothelial activation and/or damage will upset the balance between vasodilation and vasoconstriction leading to underproduction or reduce efficacy of vasodilators and/or overproduction of vasoconstrictors. While the exact mechanism for the initial endothelial injury is unknown, apoptosis induced by infection, immumed-mediated cytoxicity, antiendothelial antibodies, and ischemia-reperfusion injury have all been implicated. In addition to the imbalance of vasoactive factors, including overproduction of the vasoconstrictor ET-1 and underproduction of vasodilator NO and prostacyclin, there is proposing evidence of dysregulation of a variety of neurotransmitters and their receptors that regulate small sensory nerve fibers as sympathetic vasoconstrictor and vasodilator nerves [66]. Decreased release of vasodilatory neuropeptides from sensory nerves and up-regulation of vascular smooth muscle α_{2C}-adrenoreceptors that enhance vasoconstrictive responses to stress or cold stimuli are implicated in the dysfunction of the thermoregulatory vessels leading to RP. Patients with RP who develop SSc exhibit unique characterized

by intimal thickening that can eventually occlude the vessel lumen, thus causing ischemic injury and chronic tissue hypoxia (Figure 1.3). Skin hypoxia has been documented in SSc patients and is a potent stimulus for growth factors that mediate tissue fibrosis [10], suggesting that vascular damage is the primary insult, which then provokes tissue fibrosis.

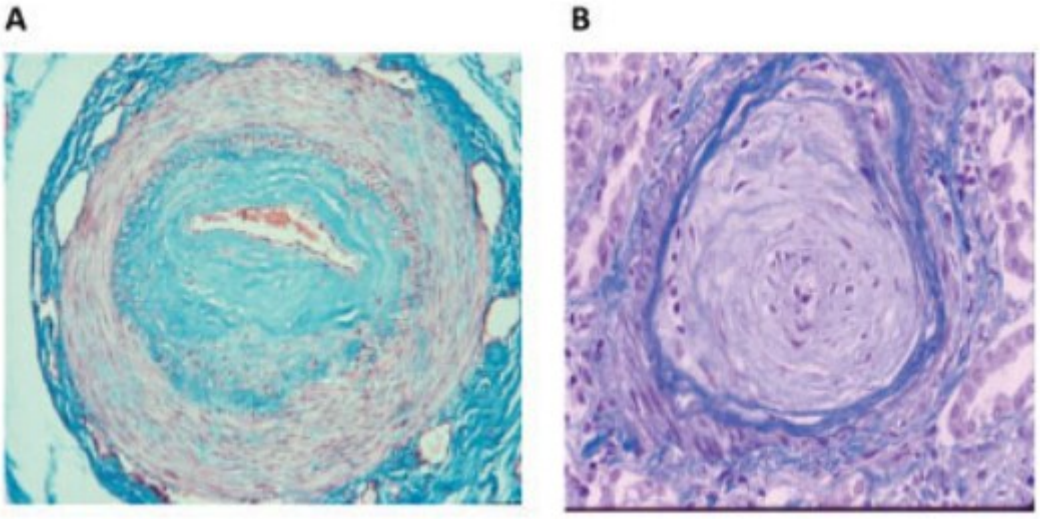


Figure 1.3. Masson's trichrome staining of a digital artery from a patient with SSc (**A**) and hematoxylin and eosin staining of a renal artery from a patient of SSc (**B**). Note of striking fibrotic intimal hyperplasia and the adventitial fibrosis in the digital artery and the onion skin-like intimal thickening composed of smooth muscle cells and increased connective tissue matrix in the renal artery. The intimal hyperplasia results in critical luminal narrowing and even occlusion [81].

1.1.4. Pulmonary arterial hypertension in systemic sclerosis

Pulmonary arterial hypertension, defined by an elevated mean pulmonary arterial pressure (mPAP) greater than 25 mmHg and a pulmonary capillary wedge pressure less than 15 mmHg, is a progressive disorder involving the pulmonary vasculature that leads to right heart failure and ultimately death [82]. PAH is a serious complication of SSc with prevalence approximately 15%. SSc- associated PAH (SSc-PAH) is a leading cause of PAH, presents

around 15-30% in all PAH. SSc-PAH has a dramatic impact on clinical course and overall survival, and remains a major cause of mortality in SSc.

In the mechanism of SSc-PAH pathogenesis, inflammation is considered as a pathological hallmark in which inflammatory cells infiltrate in pulmonary perivascular spaces within and around plexiform lesions. On the other hand, the role of growth factors such as TGF-β and VEGF in the development of SSc-PAH is also indicated in the study of Derrett-Smith *et al.* [27]. Whether this inflammatory process precedes a state of imbalance between vasoactive, growth factors and proliferative mediators leading to abnormal regulation of endothelial and smooth muscle cells as well as fibroblasts, and vascular dysfunction is not elucidated [37].

Endothelial dysfunction seems to play an integral role in mediating the structural changes in the pulmonary vasculature. Disordered endothelial cell proliferation along with concurrent neoangiogenesis, when exuberant, results in the formation of glomeruloid structures known as the plexiform lesions, which are common pathological features of the pulmonary vessels of patients with PAH. Additionally, an altered production of various endothelial vasoactive mediators, such as NO, prostacyclin, ET-1, serotonin, and thromboxane, has been increasingly recognized in patients with pulmonary hypertension. Because most of these mediators affect the growth of the smooth muscle cells, an alteration in their production may facilitate the development of pulmonary vascular hypertrophy and structural remodeling characteristic of pulmonary hypertension. It is conceivable that the beneficial effect of many of the treatments currently available for PAH, such as the use of prostacyclin, NO, and ET antagonists, result at least in part from restoring the balance between these mediators. However, the ultimate cellular and physiological targets of these treatments remain unknown [16].

In addition to the potential consequences of an imbalance in the endothelial production of various mediators, injury to the endothelium may expose the underlying vascular tissue to diverse blood-borne factors that may further promote pathological changes. Endothelial dysfunction may also have adverse consequences on pulmonary vascular hemostasis by altering the production of anticoagulant factors. Recent reports of genetic mutations in the endothelial cells of patients with pulmonary hypertension further underscore the role of these cells in the disease pathogenesis [16].

SSc-PAH shares similar histological features in IPAH, including intimal hyperplasia, medial hypertrophy, adventitial fibrosis, and inflammatory infiltrates. However, there are fewer plexiform lesions, increased intimal fibrosis, more heterogeneity, and a higher prevalence of venoocclusive lesions when compared with IPAH [45].

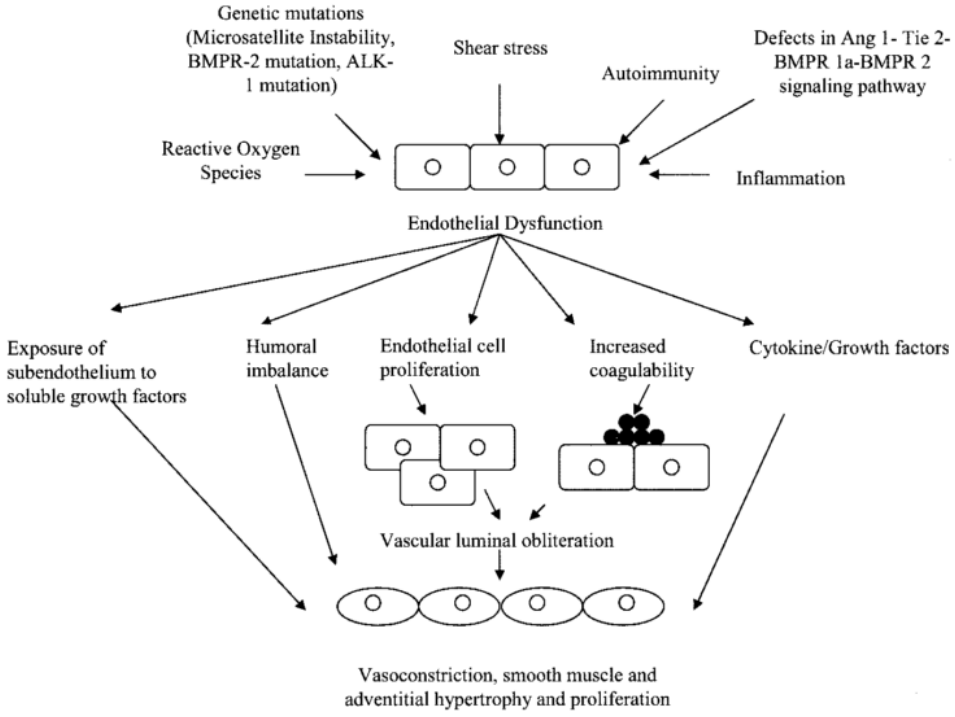


Figure 1.4. Mechanistic role of endothelial dysfunction in pulmonary hypertension and pulmonary vascular remodeling. Squares indicate endothelial cells; oval, smooth muscle cells; and closed circles, platelets [16].

1.1.5. Collagen deposition

Fibrosis represents the phenotypic expression (typified by skin thickening and interstitial lung fibrosis) of SSc. Patients with the disease have increases in collagen types 1 and 3, with type 1 being the most abundant. Type 1 collagen is encoded by the COL1A1 and COL1A2 genes, which are at least partly controlled by the transcription factor SP1. Increased SP1 binding activity has been recorded in sclerodermic fibroblasts and its activity has shown to be associated with increased gene expression of type 1 collagen in patients with SSc. Gene expression of type 1 collagen is also affected by TGF-β, which indicates a possible synergistic profibrotic interaction between SP1 and the TGF-β pathway via the SMAD3/4 complexes. Reduced amounts of SMAD7 (an inhibitor of collagen gene expression) have been reported in SSc, which suggests that the loss of this inhibitory effect allows TGF-β to stimulate unfettered, excessive accumulation of extracellular matrix.

1.1.6. Therapeutic targets in systemic sclerosis

Conventional treatments for SSc

Among the conventional therapies used for the treatment of SSc (figure 1.5), randomized trials have demonstrated a possible effect for methotrexate and extracorporeal photopheresis in improving skin thickening, as well as a role for cyclophosphamide in stabilizing or even slightly improving interstitial lung disease [24, 69]. Calcium channel blocker mostly contribute to the management of SSc peripheral vascular disease [117], while angiotensin converting enzyme (ACE) inhibitors were proven effective against renal impairment [113]. The prostacyclin analogues significantly improved pulmonary arterial hypertension associated with SSc [4] as well as peripheral vascular disease. The use of D-penicillamine as an antifibrotic agent in SSc is

not supported by the results of a randomized trial that compared high to low doses of this drug [21]. Other medications and therapeutic procedures such as cyclosporine A, sirolimius, tacrolimus, antithymocyte globulin, prazosin, and ketanserin have also been used as a therapy for SSc without achieving solid evidence of benefits [14].

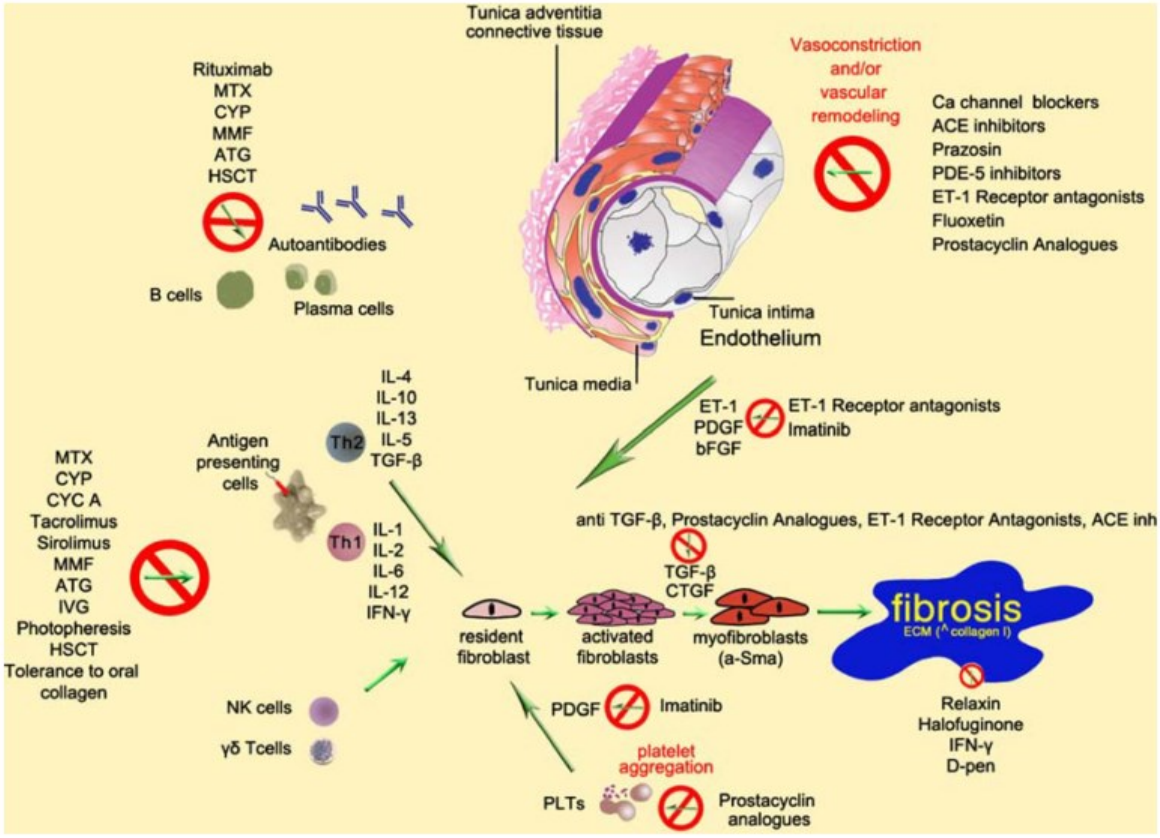


Figure 1.5. Summary of the pathogenetic processes leading to fibrosis in SSc and mechanism of action of some of the commonly used drugs. *PDGF* platelet-derived growth factor, *ET-1* endothelin-1, *IL* interleukin, *TGF-β* transforming growth factor-β, *CTGF* connective tissue growth factor, *bFGF* fibroblast growth factor, *ECM* extracellular matrix, *α-SMA* alpha-smooth muscle actin, *IFN-γ* interferon-γ, *MTX* methotrexate, *CYP* cyclophosphamide, *ATG* antithymocyte globulin, *HSCT* hematopoietic stem cell transplantation, *IVG* intravenous gamma globulin, *MMF* mycophenolate mofetil, *CYC* A cyclosporine A, *D-pen* D-penicilllamine, *PDE-5* phosphodiesterase-5, *ACE* angiotensine converting enzyme, *PLTs* platelets [14].

Therapeutic target on vascular dysfunction

As stated previously, endothelial damage and vascular dysfunction could be one of the earliest alterations in SSc [107]. The greatest advances have been in the management of vascular complications. Angiotensin converting enzyme (ACE) inhibitors has been accepted as the preferred choice in management of this rapidly fatal complication of SSc. Their mechanism of action has been well known (even in the late 1970s) and thought to be especially appropriate to treat a hypertensive state that was associated with, and driven by, elevated amounts of renin (and thus angiotensin and aldosterone) [19]. A review by Steen and colleagues, based on the Pittsburgh longitudinal database, records that 1-year survival is better than 70% in renal crisis patients treated with ACE inhibitors, but less than 20% in those not treated with ACE inhibitors.

Regarding SSc-associated PAH, some drugs have been approved by US Federal Drug Administration (FDA) due to the improved knowledge of molecular biology. Epoprostenol, treprostinil, and iloprost can supply prostacyclin that the pulmonary vascular endothelium itself no longer supplies in adequate quantities; sildenafil can increase amounts of NO in tissue; and bosetan can inhibit endothelin, which is overproduced in the serum of patients with PAH. These drugs improve the symptoms of PAH as well as the quality of life of patients with SSc-associated PAH. Increasing evidence suggests that the two treatments that have been approved the longest (epoprostenol and bosetan) could improve survival in PAH overall. Several other treatments with good molecular-based rationale have been investigated: inhaled treprostinil (a prostacyclin-like compound), inhaled nitric oxide, and other endothelin inhibitors such as sitaxsentan [46, 83]. Calcium channel blockers (especially nifedipine), intravenous iloprost and epoprostenol, and

nitroglycerin paste (oilment) are effective in lessening RP and, to a lesser extent, in healing digital ulcers. Sildenafil has also shown some effectiveness in a small placebo-controlled trial of patients with secondary RP resistant to multiple therapies [19]. A study conducted by Sfikakis *et al.* showed small doses of bosentan improve endothelial function without affecting hemodynamic parameters or endothelial activation-related processes, thus supporting a direct, reversible effect of endothelin in SSc-associated vascular injury [110].

Iloprost in vasodilator therapies

The digital arteries of patients with SSc exhibit marked fibrotic intimal hyperplasia and luminal narrowing. Normal vasoconstrictor responses to cold, superimposed on this anatomic obstruction, could cause occlusion of the arterial lumen. Vasodilators that inhibit cold induced vasoconstriction might prevent this vessel closure. Alternatively, vasodilator therapies might be predicted to be minimally effective in the setting of fixed vascular obstruction [29].

Iloprost is a stable analogue of prostacyclin that is associated with a longer duration of vasodilatation [38]. It is widely used for the treatment of vascular dysfunction in systemic sclerosis due to its well-known vasodilator and anti-aggregant effect [36].

According to the recommendations of the European League against Rheumatism (EULAR) for the treatment of systemic sclerosis, iloprost is effective in reducing the frequency and severity of SSc-RP. Iloprost, given intravenously (0.5–3 ng/kg per minute for 3–5 consecutive days sequentially) or orally (50–150 mg twice a day) significantly reduced the frequency of ischaemic attacks, and improved the RP severity score in comparison with

placebo. Oral prostanoids seem to be generally less effective than intravenous iloprost in the treatment of SSc-RP, although some beneficial effects could be seen with higher doses. Intravenous prostanoids (particularly intravenous iloprost) are efficacious in healing digital ulcers in patients with SSc. Intravenous prostanoids (in particular iloprost) should be considered in the treatment of active digital ulcers in patients with SSc [71].

Iloprost may promote repair of damaged endothelium, which could explain vascular effects and clinical improvement weeks after [29]. Dole *et al.* showed that iloprost can improve healing of cutaneous lesions including ischemic ulcers in patients with SSc. The ischemic lesions occur because of a vasculopathy characterized histologically by abnormal vascular endothelium and intimal thickening, and clinically by vasopasm. The benefit of iloprost on tissue healing may in part be explained by the potential capacity of prostacyclin to inhibit platelet activation and leukocyte adherence to the endothelium thus reducing counteracting defective endothelial function and tissue injury [29].

1.2. Oxidative stress and systemic sclerosis

Reactive oxygen species (ROS) is an expanded term from free radicals which have been defined as species capable of independent existence that contains one or more unpaired electrons in atomic or molecular orbitals [91]. These unpaired electrons make free radicals highly reactive in which superoxide radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$), hypochlorous acid (HOCl) and peroxynitrite ($ONOO^-$) are common oxidative molecules in the ROS family [87]. With excessive levels, ROS are addressed oxidative stress and cause cellular damage and cell transformation**.**

Several sources of ROS mentioned to contribute to oxidative stress in fibrotic processes of SSc. Ischemia-reperfusion is a main cause leading to tissue

damage. In addition, the interaction of cytokines and growth factors with their receptors can induce ROS in SSc. Among those, TGF-β is a pivotal profibrotic cytokine, which expressed the key role in pathogenesis of SSc. Some other significant factors involved are platelet derived growth factors (PDGF), vascular endothelial growth factor (VEGF) [12], connective tissue growth factor (CTGF), angiotensin II, interleukin 3, interleukin 6, tumor necrosis factor-alpha (TNF-α), nerve growth factor, fibroblast growth factor. Angiotensin II, PDGF and TGF-β1 can interact with members of the nicotinamide adenine dinucleotide phosphate oxidase (NOX) family in vascular smooth muscle cells, cardiac, lung and skin fibroblasts [42]. The increased level of superoxide from fibroblasts and monocytes also contributes to abundant sources of ROS in SSc [30].

RP occurring in almost all SSc patients, approximately more than 90% [58], presents the oxidative pathway beside the nonoxidative pathway in pathology of this phenomenon associated with SSc [51]. There is evidence showing induced effects on SSc-associated Raynaud's phenomenon. The level of 8-isoprostane, a reliable biomarker of oxidative stress and antioxidant deficiency, has been shown as a valid measure of lipid peroxidation *in vivo* [89]. It is correlated with the extent of vascular lesion in RP and the severity of fibrosis in SSc patients [94, 114]. Free radical nitric oxide (NO) released by endothelial cells is considered as a beauty in the protective role in controlling vasculature and regulating blood pressure as well as acting as antithrombotic and cytoprotective agent. However, in RP, during reperfusion, free radials and NO produced overwhelmingly lead to peroxynitrate which precedes oxidative vascular damage and endothelial apoptosis, therefore NO is considered as a beast [17, 51].

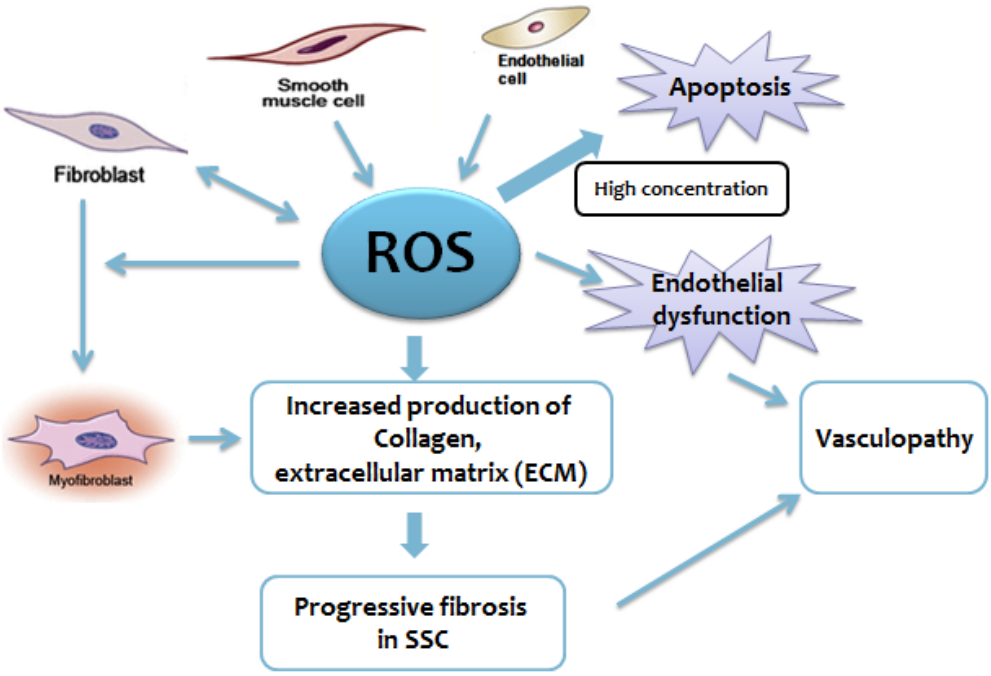


Figure 1.6. Schematic diagram of ROS-induced fibrotic process in SSc.

Abundant studies have shown the relevance of ROS in pathogenesis of SSc *in vitro* and *in vivo* [57, 94, 105, 109]**.** Oxidative stress is considered as one of the main background mechanisms contributing to the progress and destruction of this disease, concomitantly clinical manifestations regarding to SSc [51]**.** Skin and visceral fibroblasts spontaneously produce large amounts of ROS that initiate collagen synthesis [51]. Recently, a preliminary study of Boin et al showed substantially increased intracellular ROS levels in human pulmonary artery smooth muscle cells (HPASMCs) treated by sera of SSc patients with pulmonary artery hypertension (PAH). Employing NOX2ds-tat (gp91ds-tat), a specific inhibitor of NOX2, can effectively reduce PAH-SSc sera -induced ROS, implicating the involvement in the mechanism of increasing ROS of SSc. Exposure of HPASMCs to SSc-PAH sera also resulted in progressive time-related increase of the Collagen promoter activity. Similarly, this effect was inhibited by NOX2ds-tat treatment, suggesting the collagen synthesis activation in HPAMSCs may be driven by

SSc-related PAH sera through NADPH oxidase-dependent ROS generation. Taken together, NADPH oxidase-derived ROS mediates the activation of collagen synthesis [13]. *In vivo* studies, exploiting BALB/c mice, the SSc murine model injected HOCl every day for 6 weeks induced chronic oxidative stress. As a result, this HOCl-induced oxidative stress operated cutaneous and lung fibrosis in SSc mice. Furthermore, HOCl-treated mice also overexpressed α smooth muscle actin (α-SMA), a biological marker characterized by activated myofibroblasts. These processes occurred through ADAM17/Notch pathway or Ras-ERK pathway or guanosine triphosphate (GTPase) intracellular signaling [5, 6, 68]. Blocking the activation of the Ras-ERK pathway by propylthiouracil (PTU) or simvastatin can prevent the pulmonary fibrosis and cutaneous fibrosis in oxidant-stress animal model of SSc [5, 6].

Some studies reported that anomalous overproduction of ROS activates fibroblasts linking to the increased expression of stimulatory serum autoantibodies to the PDGFR in SSc [8]. While prooxidants may cause the surging of autoantibodies, other studies showed the uncorrelated relation between autoantibodies in endothelial cells and fibroblasts and serum-induced ROS or cell proliferation. Nonetheless, it is undeniable the role of autoantibodies in the vast damage in SSc in which anti-endothelial cell antibodies (AECA) were closely correlated with pulmonary fibrosis with SSc [51, 61]. Oxidative stress may either directly activate ROS-induced differentiation of fibroblasts into myofibroblasts [68] or disrupt the balance between protease and protease inhibitors or both. With the latter mechanism, on the one hand, TGF-β upregulates the expression of extracellular matrix proteins including collagens, on the other hand, TGF-β suppresses protein degradation through enhancing activities of protease inhibitors such as

plasmin activator inhibitor 1 (PAI-1) and tissue inhibitors of metalloproteinases (TIMPs) [77]. This protease-antiprotease imbalance is likely to be a critical mechanism in the development of SSc lung fibrosis [43].

1.3. Role of endothelial NADPH oxidases in SSc

NOXs are the only enzymes with the primary function of generating ROS [31, 73]. Other enzymes (cyclooxygenases, cytochrome P450, enzymes of the mitochondrial electron transport chain) can produce ROS, but only as a byproduct of their normal function. Of note, these latter enzymes only become ROS-generators in the presence of an external source of ROS (such as NOXs) and can thus be considered as secondary sources of ROS [32]. NOXs catalyze the transfer of electrons from cytosolic NADPH to molecular O2 via their membrane-bound catalytic NOX or DUOX subunit to generate the ROS, superoxide or H_2O_2. NOX-derived ROS may promote ROS formation from other (normally dormant) enzyme sources, setting up a vicious cycle that further exacerbates vascular oxidative stress [78, 86, 92]. Accumulative evidence highlights the involvement of NADPH oxidase-dependent redox signaling in the profibrotic responses mediated by TGF-β [104].

Seven isoforms of NOX have been described in mammals, each distinguished mainly by the identity of its catalytic subunit. They are the 'NOXs': NOX1-, NOX2- [formerly gp91phox; also known as CYBB (cytochrome b245, b polypeptide)], NOX3-, NOX4-, and NOX5-containing oxidases; and the 'DUOXs' ('dual oxidases'): DUOX1- and DUOX2-containing enzymes. Four of the NOXs – NOX1, NOX2, NOX4, and NOX5 – are expressed in endothelial cells.

Of the four endothelial NOXs, three (NOX1, NOX2, and NOX4) are known to exist as multimeric protein complexes, whereby electron transfer occurs through the 'NOX' subunit in association with different combinations of regulatory subunits. Figure 1.7 illustrates the molecular structures of the endothelial NOXs.

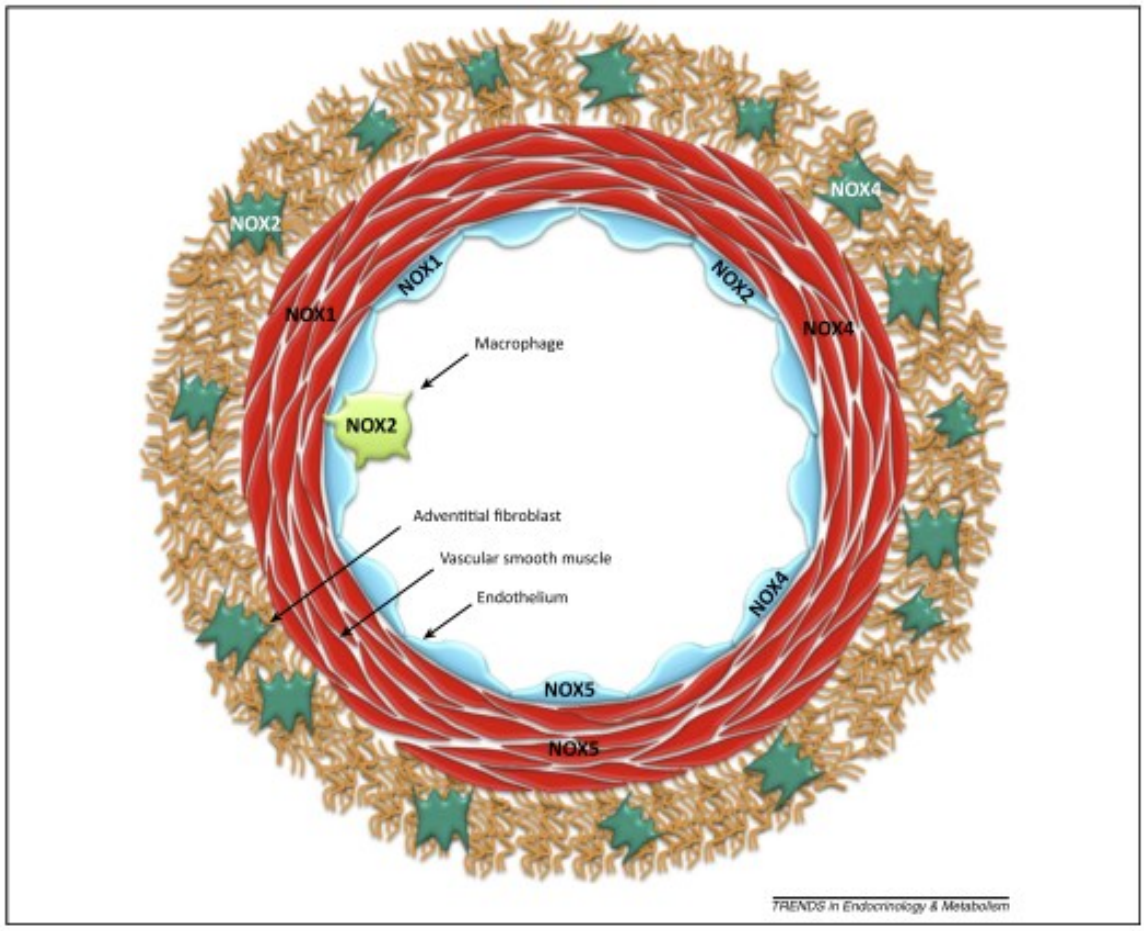


Figure 1.7. NADPH oxidase (NOX) isoform expression in the various cell types of the blood vessel wall. Four NOX isoforms are expressed in the vascular wall, including NOX1 (in endothelial cells and vascular smooth-muscle cells, VSMCs), NOX2 (in endothelial cells, adventitial fibroblasts, and leukocytes such as monocytes, macrophages, and platelets), NOX4 (in endothelial cells, VSMCs, and adventitial fibroblasts), and NOX5 (in endothelial and VSMCs – not expressed in rodents) [32].

NOX2 was the first NOX isoform to be identified in endothelial cells and, as shall be discussed below, is likely to be the most important in the context of vascular pathology.

NOX4 is the most highly expressed NOX homolog in endothelial cells. It also requires p22phox, but apparently does not associate with Rac1 or any of the cytosolic organizer or activator proteins needed for activation of NOX1 and NOX2 [32]. Current evidence suggests that Nox4 may have a pivotal role in mediating TGF-β-induced profibrotic responses [104]. TGF-β increased Nox4 gene expression without effects on Nox1, Nox2, or Nox5 in human cardiac fibroblasts [22]. Treatment with siRNA against Nox4 suppressed expression of TGF-β target genes including fibronectin, collagen I, α-smooth muscle actin (α-SMA, a marker of myofibroblast), and connective tissue growth factor, indicating that Nox4 was involved in TGF-β-induced differentiation of cardiac fibroblasts to myofibroblasts [22]. In renal tubular epithelial cells, Rhyu *et al.* showed that inhibition of NADPH oxidase activity with the non-selective Nox inhibitor DPI suppressed EMT and matrix protein production [100].

NOX5 appears to be the only endothelial NOX that can produce ROS in the absence of other 'phox' or Rac subunits. NOX5 is also unique among NOX isoforms in that it contains an N-terminal calmodulin-like domain with four binding sites for Ca^{2+} (EF hands). Thus, endothelial NOX5 activity can be directly modulated by changes in intracellular [Ca^{2+}] [9].

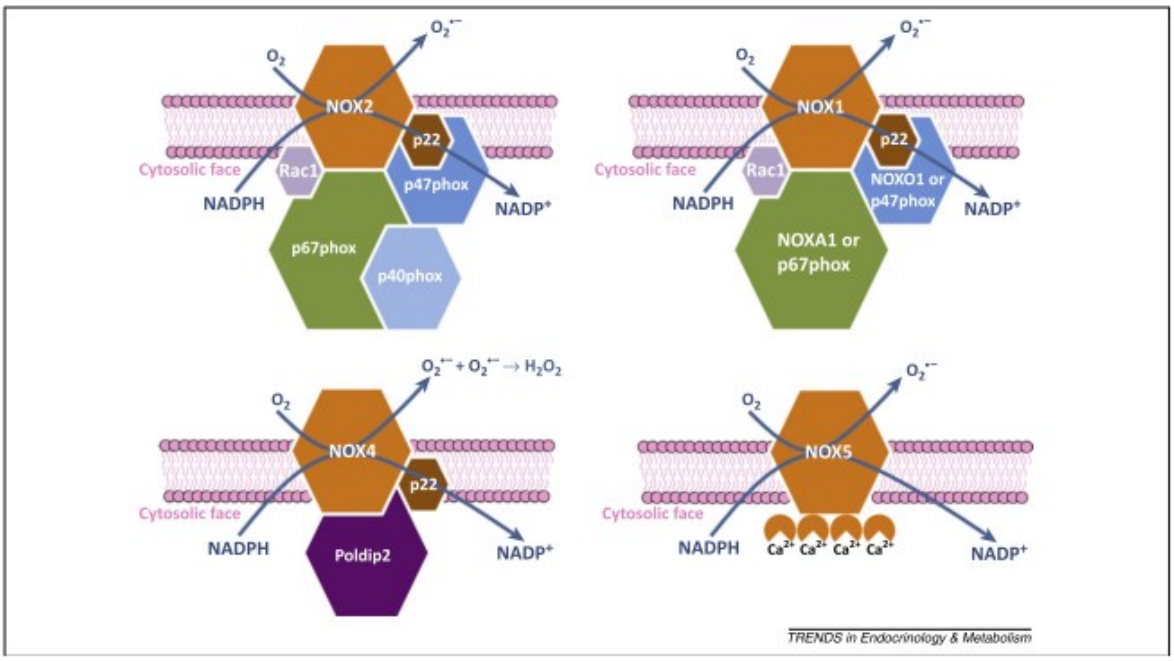


Figure 1.8. Molecular composition of the endothelial NADPH oxidases ('NOXs'). All four endothelial NOX isoforms are comprised of a catalytic 'NOX' subunit, which may reside in the nuclear, endoplasmic reticulum (ER), or plasma membranes. For NOX1, 2, and 4, additional subunits are required for full enzyme activity. By contrast, NOX5 is a "stand alone" protein whose activity is regulated by Ca^{2+} binding. The topology of the NOXs is such that NADPH binds to the cytoplasmic domains of the proteins with reactive oxygen species (ROS) generation always occurring on the opposite side of the membrane (i.e., within the nucleus, ER, or the extracellular compartment). It is also noteworthy that whereas NOX1, 2, and 5 generate superoxide, NOX4 appears to generate hydrogen peroxide [32].

1.4. Endothelial-to-Mesenchymal Transition and Systemic Sclerosis

Pathogenesis of systemic sclerosis constitutes numerous complex mechanisms associated with (i) microvascular fibroproliferative lesions, (ii) innate and adaptive immune system abnormalities and uncontrolled accumulation of collagen, (iii) other extracellular matrix compartments produced by

fibroblasts and activated myofibroblasts in skin and various internal organ [64, 95]. In a non-pathological condition, myofibroblasts usually disappear through apoptosis and through transition to a quiescent/senescent state at the later stages of wound healing. However, the persistence of myofibroblasts under an activated phenotype in EndMT contributes to progressive fibrogenesis [90, 111]. The consequence of this process is causing interstitial and perivascular fibrosis in the lungs, heart, kidneys and other organs responsible for the high mortality of SSc patients [11]. The origin of activated myofibroblasts in SSc has been demonstrated from various sources including pericytes and smooth muscle cells (SMCs) from vessel walls, resident fibroblasts, bone marrow-derived fibroblasts [60, 64, 126]. With large amounts of studies, Epithelial-to-Mesenchymal Transition is a well-known source of activated myofibroblasts by which epithelial cells transforms into myofibroblasts. Although it is not extensively studied as EMT, more recent research has been conducted to investigate the mediators and signaling pathways in mechanisms of differentiation of endothelial cells to mesenchymal transition. EndMT is considered as a special form of EMT, vascular endothelial cells share several similar characteristics and molecular mechanisms with epithelial cells to generate fibroblasts and myofibroblasts [74, 99]. EndMT contributes to fibrotic pathogenesis of cardiac fibrosis, pulmonary fibrosis, renal fibrosis, idiopathic portal hypertension, liver fibrosis, and obviously systemic sclerosis [54, 64, 106, 115, 127].

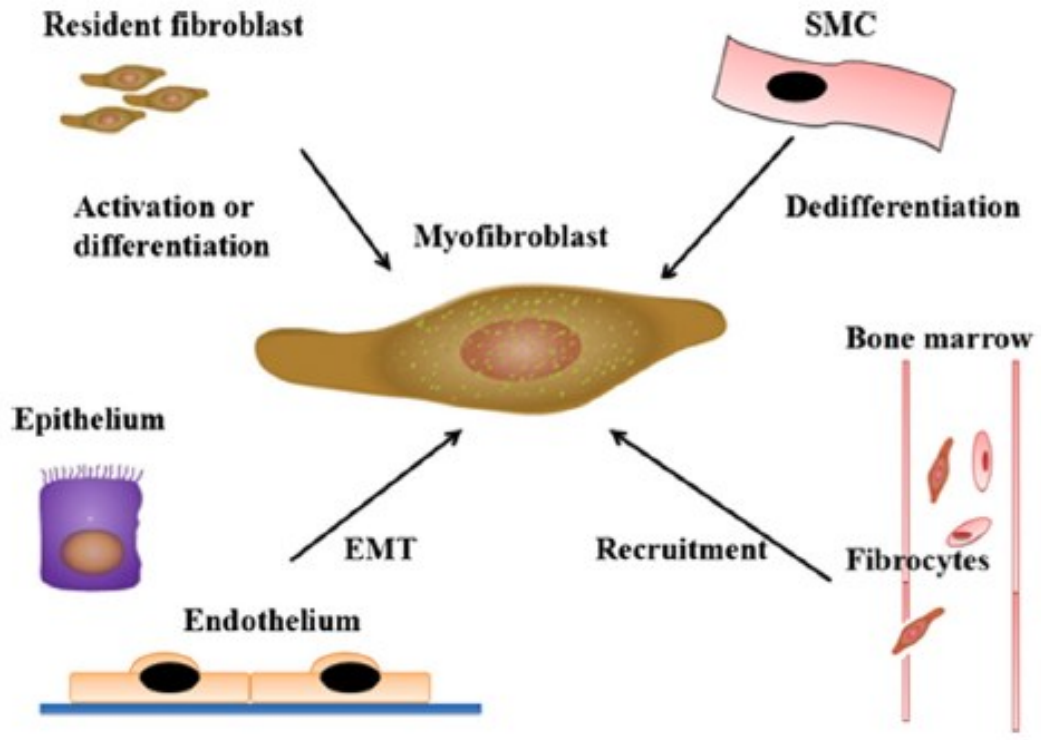


Figure 1.9. Multiple origins of myofibroblasts [126].

Under the conversion of endothelium into mesenchyme, endothelial cells undergo morphological alterations and loss of cell-surface markers. In cell culture, endothelium acquires mesenchymal, fibroblast-like properties such as spindle-shaped morphology, migratory capacity, invasiveness, and enhanced resistance of apoptosis [60, 120]. During EndMT process, the structure of vessel-lining is disrupted due to resident endothelial cells disaggregating from the organized layer of cells on vessel walls and invading the surrounding tissue [76, 97, 120]. Cell-surface markers like vascular endothelial cadherin (VE-Cadherin), CD31 (platelet endothelial cell adhesion molecule-1, PECAM 1) or von Willebrand factor (vWF) are gradually replaced by markers characterized for mesenchyme such as fibroblast specific protein-1 (FSP-1), α-SMA, vimentin and type I and type III collagen [54, 97].

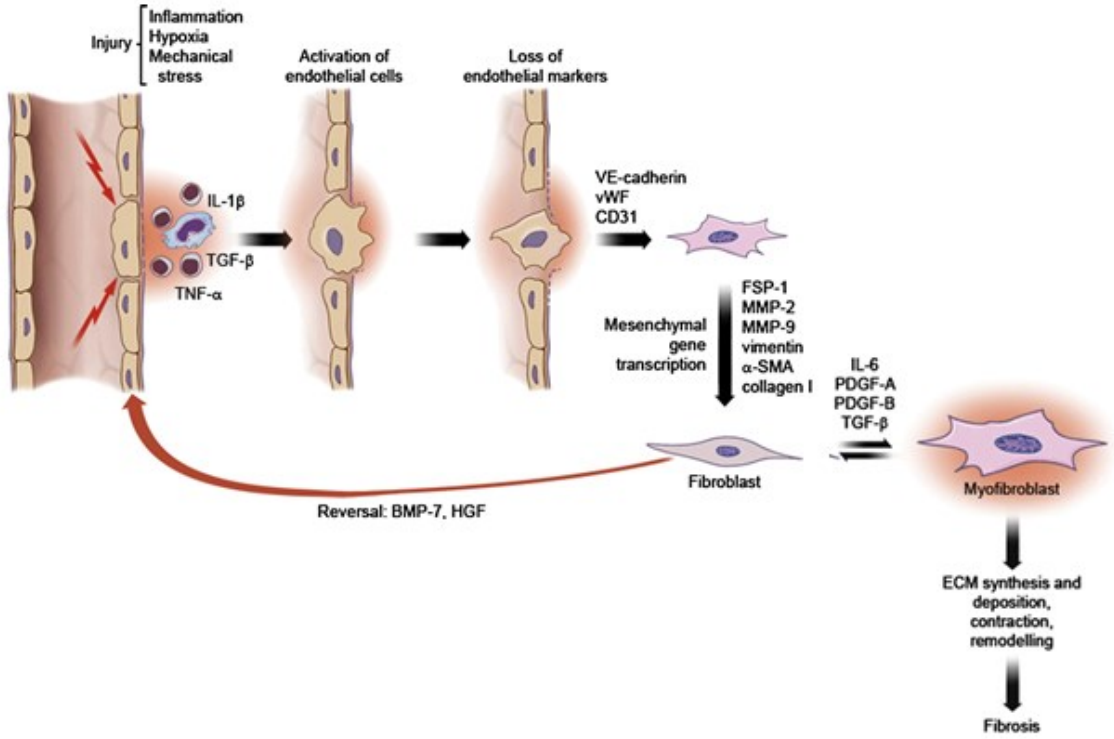


Figure 1.10. Endothelial-to-Mesenchymal Transition. Endothelial cells can transdifferentiate into fibroblasts under the influence of several factors. This transition is accompanied by the progressive loss of typical endothelial cell markers (VE-cadherin, vWF, CD31) and the transcription of typical mesenchymal cell markers (FSP-1, MMP-2, MMP-9, vimentin, α-SMA, collagen I). This process can be reverted by the administration of BMP-7 or HGF. Under the effect of variety of mediators, fibroblasts differentiate into myofibroblasts which leads to ECM and collagen synthesis [102].

Studies demonstrated the presence of transitional EndMT cells in the pulmonary vasculature of patients with SSc-PAH. The expression of cell surface markers specific for endothelial cells in mesenchymal/fibroblastic cells or vice versa from SSc-PAH patients [48, 97]. Treatment of IL-1β, TNF-α and TGF-β in combination abolished cobblestone structural characteristics and enhanced spindle-like appearance on pulmonary artery endothelial cells. Induced endothelial-mesenchymal transition (I-EndMT) cells exhibit an elevated secretion of proinflammatory proteins and collagen type I. In addition, the presence of I-EndMT cells in cellular barriers leads to substantially

increased paracellular and transcellular permeability which is considered as one of the earliest signs of vascular dysfunction in SSc [43, 48].

The mechanism of EndMT in pathogenesis of SSc vasculopathy is postulated to be induced by synergistic and complex activation of a large variety of cell types exposing to locally various biological mediators, such as inflammatory cytokines, growth factors [54, 95]. However, the precise molecular mechanisms of EndMT process mediated by those mediators and signaling pathways have not been entirely illuminated. Among these mediators, TGF-β has been implicated as a key role in the initiation of EndMT by numerous evidence [23, 49, 64, 90, 97, 120].

TGF-β can either solely activate endothelial cells or synergize with TNF-α and IL-1β to induce EndMT. Maleszewska et al (2013) demonstrated that the treatment with either TGF-β2 or IL-1β resulted in alterations of endothelial cells morphology, followed by a spindle-shaped phenotype. Nevertheless, under a co-stimulation condition the morphological changes were more explicit than in the case of single-factor stimulation [79]. Similarly, Good et al (2015) showed evidence that a combination of TGF-β with IL-1β and TNF-α stimulated the induction of EndMT in pulmonary artery endothelial cells. Notably, the withdrawal of IL-1β, TNF-α, and TGF-β after 6 days failed to convert induced EndMT (I-EndMT) cells to normal structural characteristic. It indicated that phenotypic change was permanent and was not reversible [48, 99]. This observation was in agreement with previous studies that EndMT would be consistent in the cells exposed to activated Ras and TGF-β treatment [53]. However, other reports showed that some anti-fibrotic mediators could dedifferentiate established myofibroblasts. This raises enormous hope for regenerative medicine in the reversal of the EndMT

process, which provides a new intriguing therapeutic target for fibrotic diseases [44, 126].

There are some signaling pathways involved in EndMT in which TGF-β plays a critical role. Both Smad-dependent and Smad-independent pathways are associated in intracellular cascades activated by TGF-β during endothelial-mesenchymal transition. Smad proteins have been shown to bind directly to the promoter of the Snail gene to regulate its transcription [23] . Snail-1, a transcriptional repressor, is crucial for TGF-β-induced mesenchymal transdifferentiation of embryonic stem cell-derived ECs. Snail-1 is a zinc-finger transcription factor that forms a complex with Smad3/Smad4. The active Smad3/Smad4/Snail-1 complex results in potent inhibition of the expression of E-cadherin by directly integrating into specific sequences within the gene promoter and blocking its transcription. In addition to inhibition of E-cadherin, Snail-1 precedes transcriptional events that lead to the expression of a mesenchymal-cell-specific phenotype [64]. In the Smad-independent pathway of TGF-β signaling, there is an involvement of important kinases such as c-Abl protein kinase (c-Abl), protein kinase Cδ (PKC-δ), and glycogen synthase kinase 3β (GSK-3β). GSK-3β is a crucial factor, whose phosphorylation will cause its inhibition and induce the strong up-regulation and subsequent translocation of Snail-1 into the nuclear. In the end, this process increases the expression of mesenchymal cell-specific marker such as α-SMA, type I collagen while reduce the expression of VE-cadherin characterized for endothelial cells. Using inhibitors of c-Abl and PKC-δ, imatinib and rottlerin respectively can inhibit the phosphorylation of GSK-3β, hence; abolish the transdifferentiation of ECs into myofibroblasts. This intervention could be a potential therapeutic target to obliterate the acquisition of the myofibroblastic phenotype through EndMT process [75].

TGF-β2 signals through MEK, PI3K, and p38MAPK pathways are also essential for ECs undergoing EndMT transition [84]. In addition, regulation of EndMT by Wnt [123] and NOTCH signaling [93], Caveolin-1 signaling [25, 26] is indicated to precede endothelial-mesenchymal transition regarding to TGF-β activity.

Other cytokines and growth factors might also play important roles in endothelial-mesenchymal transition such as PDGF, VEGF, endothelial-1 (ET-1), CTGF, MicroRNAs (miRNAs) [64].

1.5. Reactive oxygen species and Endothelial-to-Mesenchymal Transition

With numerous profound studies of the effects of oxidative stress on endothelial dysfunction, ROS have been highlighted in the process of epithelial-mesenchymal conversion [88]. Previous reports have demonstrated that EMT is closely relevant to ROS [74]. Recently, it has been reported that oxidative stress induces the EMT in renal tubular epithelial cells, human epithelial keratinocytes, and lung epithelial cells [28, 40, 101]. An emerging issue is whether there is a similar tendency towards EndMT.

1.5.1. ROS activate/mediate TGF-β-dependent signaling pathways in EndMT.

TGF-β is a multifunctional protein including three isoforms (TGF-β1, TGF-β2, and TGF-β3) which accounts for the regulation of cell proliferation, differentiation, apoptosis, adhesion, and migration. It therefore shows profound systemic effects on physiological functions [63, 77]. TGF-β stimulates the generation of ROS in various types of cells while ROS activate TGF-β and mediate effects of TGF-β. TGF-β elevates ROS production via NOX4, mitochondria, and microsomes. On the other hand, ROS induce TGF-β gene expression and activate TGF-β through oxidizing latency association

protein (LAP) or activating MMPs, which in turn release LAP [77]. It is well known that TGF-β is synthesized as a non-active pro-form combining with latency association protein (LAP) to create a latent complex. ROS oxidizing Redox center of LAP can trigger a conformational change resulting to the release of TGF-β1 [65]. Hence, under the stimulation of ROS, TGF-β is activated and increasingly expressed.

It is well documented that TGF-β induced EndMT process in cardiac, pulmonary fibrosis, renal, intestinal and skin fibrosis [20, 53, 54, 103, 127] whereas NADPH oxidases and ROS molecules may have been indicated to play a pivotal role in mediating TGF-β induced fibrotic responses via Smad2/Smad 3 activation [63]. Of note, NOX4-dependent generation of H_2O_2 is required for TGF-β1-induced myofibroblasts differentiation and elevated ECM production [55, 62].

A recent study of Montorfano group used human umbilical vein endothelial cells (HUVECs) to investigate the role of ROS as well as the underlying mechanism in the conversion of ECs into myofibroblasts. They demonstrated that oxidative stress is a crucial factor in generating the phenotypic conversion of ECs into activated myofibroblasts with an EndMT-like mechanism via a TGF-β1 and TGF-β2-dependent pathway. In addition, this study showed that ROS-induced oxidative stress acted on the conversion through the ALK5/Smad3/NF-κB intracellular pathway. The main point of this study supporting the hypothesis that ROS, TGF-β and EndMT is in an interactional relation is H_2O_2 induced the expression and secretion of TGF-β1 and TGF-β2, which has been demonstrated to induce the conversion of ECs into myofibroblasts [87]. Another recent study provided evidence that lipopolysaccharide-induced ROS could lead to an EndMT-like process through an ALK5 activity-dependent mechanism [33]. In line with this

observation, Toshio group (2015) also showed novel evidence that endotoxaemia-derived oxidative stress might be one of the promoters of TGF-β-mediated EndMT in pulmonary vascular endothelial cells [118]. This evidence was strongly supported with the newer study by the same authors that the expression of TGF-β1 and TGF-β2 is crucial for the development of the endotoxin-induced endothelial fibrosis mechanism. This increase of endotoxin-induced TGF-β1 and TGF-β2 expression required the activation of NOX and the subsequent generation of ROS [34]. Taken together, oxidative stress is likely a link to mediate the EndMT process induced by TGF-β.

1.5.2. Oxidative stress and EndMT in SSc: is there a link?

Despite the fact that the role of ROS has been well defined in many interstitial organ fibrosis as well as strong there being strong evidences displaying the close relationships between ROS and SSc, EndMT and SSc (stated above) via numerous relevant papers, few studies have conducted experimental research indicating the effects of oxidative stress on EndMT process in SSc. A study conducted by Hao Xu group showed chronic oxidative stress mediates Endothelial-to-Mesenchymal Transition in a murine model of SSc [125]. They isolated microfibrils from the skin of tight skin ($Tsk^{+/-}$) mice, which showed abnormal big fibrillin-1. EC cultures on this abnormal extracellular matrix leaded to the alterations of morphology and function of ECs, and increased 4-HNE, a well-known fission product of polyunsaturated fatty acid oxidation. There was a transdifferentiation from ECs to mesenchymal cells with the increased presence of FSP-1 and Twist (a transcription factor implicated the endothelial cell to fibroblast transition) and a decrease of VE-cadherin. Beside, abnormal big fibrillin expression was associated with oxidative stress (reduced nitric-oxide-to-superoxide anion ratio). This suggested an oxidative involvement in the transition. In addition, pretreatment

D-4F, a peptide binding with high affinity to oxidized lipids attenuated or abolished EndMT. This indicated that the oxidized lipids might play a central role in the mechanisms by which other chronic states of oxidative stress promotes endothelial mesenchymal transition [125]. In this paper, the authors did not mention TGF-β, which has been well documented to be a crucial role in the initiation of EndMT during various diseases [64]. Of note, ECM including fibrillin functions as a reservoir for TGF-β and other growth factors to control mesenchymal differentiation [121]. Whether there is an interaction between TGF-β and oxidative stress to precede EndMT in this case is an intriguing question, which requires more investigation to figure out.

2. RESEARCH OBJECTIVES

Systemic sclerosis (scleroderma, SSc) is a complex multisystem autoimmune disease characterized by dysregulated immune system, progressive fibrosis of skin and visceral organs in which extensive vascular alterations are prominent and severe [41].

Despite of the enormous scientific effort in studying etiology and pathogenesis of scleroderma, an overall picture of its etiopathogenic mechanisms remains obscure. Oxidative stress-mediated vascular dysfunction is one of the important features of the detrimental vascular changes in SSc patients. Although the presence of a large excess of ROS has been supported by numerous studies, which documented the aberrant redox state and demonstrated the involvement of ROS in excessive extracellular matrix deposition, the implication of molecular machinery is still unclear [42]. Many clinical trials with antioxidants have been undertaken to inhibit the progression of SSc.

Iloprost, a stable prostacyclin analogue with vasodilating, anti-platelet, cytoprotective and immunomodulating properties, has been found to be an efficacious alternative to nifedipine for the treatment of SSc [108]. Some studies reported that iloprost infusion reduces oxidative stress and improves fibrosis in SSc patients [36, 108, 116]. However, the mechanism underlying this beneficial effect remains unelucidated.

Aside from Epithelial-to-Mesenchymal Transition (EMT), which has been extensively shown to occur in a variety of fibrotic diseases including SSc [18, 54, 70, 115], a potential role of Endothelial-to-Mesenchymal Transition (EndMT) in scleroderma has been indicated in some reports. EndMT may trigger the enhanced fibroproliferative vasculopathy and is considered as a

novel mechanism for the generation of activated myofibroblasts in SSc [64]. Consonant to what reported above, ROS mediate TGF-β-induced differentiation of myofibroblasts, which accounts for the increased production of fibrillar type I and type III collagen and other extracellular matrix (ECM) proteins, and triggers the expression of α-smooth muscle actin (α-SMA), a process strongly correlated EndMT [22, 64].

The research question of this study was to understand whether oxidative stress and EndMT could be part of the molecular machinery inducing vascular damage in SSc patients.

Therefore, we have been conducting this study with the following intents:

1. To investigate whether SSc sera can induce oxidative stress and collagen synthesis in HPMECs.

2. To examine the effect of intravenous iloprost infusion on SSc sera and how this alteration affects in HPMECs.

3. To investigate whether SSc sera can induce Endothelial-to-Mesenchymal Transition in HPMECs.

3. MATERIALS AND METHODS

3.1. Ethical approval

The study was approved by the Ethics Committee, from both the University of Sassari and the John Hopkins University, Baltimore (USA), and all patients enrolled were informed consent.

3.2. Patient selection

Serum samples were collected from patients with SSc at the Johns Hopkins Scleroderma Center, Division of Rheumatology, Johns Hopkins University, Baltimore, and at the Unit of Complex Rheumatology, Sassari. In Baltimore, nineteen patients were enrolled in the study. In Sassari, sixteen patients were enrolled in the study. The clinical and serological characteristics of these SSc patients in Baltimore and Sassari are summarized in table 4.1 and table 4.2, respectively.

All the patients with SSc had to fulfill the American College of Rheumatology (formerly, the American Rheumatism Association)-SSc classification criteria. Using these guidelines, patients with skin sclerosis limited to hands, forearms, legs below the knee, and face were defined as having limited scleroderma. Those with more extensive skin disease spreading proximal to elbows or knees or involving the trunk were defined as having diffuse scleroderma. The onset of the disease was defined as the time of the first non-Raynaud phenomenon.

The SSc patients from Sassari underwent a monthly intravenous administration by an infusion pump of the prostacyclin analogue iloprost, at the greatest tolerated dose (0.5-2 ng/kg/min, mean dose 1.3 ng/kg/min), for 5-6 hours/day. Blood samples were taken at two different time points. For each

patient, blood samples were taken at baseline and 5 hours after the cycle of iloprost infusion. Samples were stored at -80^{0}C until being assayed.

Previous medications were maintained during the course of the study; no other drugs were started during the study period.

Healthy donors were matched for gender, race, and smoking status.

3.3. Cells and Chemicals

Human pulmonary microvascular endothelial cells (HPMECs) were supplied by Innoprot, S.L, Spain.

Primary antibodies Rabbit polyclonal IgG anti-von Willebrand (Santa Cruz Biotechnology, INC); Goat Polyclonal Anti-Actin Alpha 2 Smooth Muscle Antibody (Novus Biologicals); β-actin antibody from Sigma-Aldrich (Saint-Louis, MO, USA); the HRP-conjugated secondary antibodies were from Santa Cruz Biotechnology, INC and Pierce Biotechnology-Thermo Scientific (Rockford, USA).

3.4. Cell culture

Human pulmonary microvascular endothelial cells (HPMECs) were cultured in endothelial cell medium (ECM) (Innoprot, S.L, Spain) supplemented with 5% of Fetal Bovine Serum (FBS), 1% of Endothelial Cell Growth Supplement (ECGS), and 1% of Penicillin/Streptomycin solution (P/S solution). The cells were maintained at 37^{0}C and 5% CO_2 in a humid environment.

3.5. Measurement of intracellular ROS

Intracellular ROS levels were determined by using the ROS molecular probe 2', 7'-dichlorodihydrofluorescein diacetate (H_2-DCFDA) (Molecular Probe, Eugene, OP) as previously described with minor modification. Within the cell, esterases cleave the acetate groups on H_2-DCFDA, thus trapping the

reduced form of the probe (H_2-DCF). Intracellular ROS oxidize H_2DCF, yielding the fluorescent product, DCF.

HPMECs (10^5 cells per well) were seeded in black 96-well plates (Costar, Corning, Inc, NY) and incubated with their respective growth medium alone for 24 hours at 37^0C with an atmosphere of 5% CO_2/95% air. Culture media were then removed and cells were pre-incubated for 15 minutes with Earle's balanced salt solution (Sigma, MO, USA) containing 10 μM H_2-DCFDA, and then washed with PBS. After 15 minutes, cells were treated with sera (10% V/V) of SSc and HD. Fluorescence was measured by using a Tecan GENios plus microplate reader (Tecan, Mannedorf) in a light protected condition for 5 hours. Excitation and emission wavelengths used for fluorescence quantification were 485 nm and 535 nm respectively.

3.6. Measurement of intracellular ROS with NADPH inhibitors

HPMECs (10^5 cells per well) were seeded in black 96-well plates (Costar, Corning, Inc, NY) and incubated with their respective growth medium alone for 24 hours at 37^0C with an atmosphere of 5% CO_2/95% air. Cells were pre-treated with a broad NOX inhibitor, diphenylene iodonium (DPI) 5μM for one hour or with a specific NOX2 inhibitor, gp91ds-tat 5μM for one hour. Culture media were then removed and cells were pre-incubated for 15 minutes with Earle's balanced salt solution (Sigma, MO, USA) containing 10 μM H_2-DCFDA, and then washed with PBS. Subsequently, cells were treated with sera (10% V/V) of SSc and HD. Fluorescence was measured by using a Tecan GENios plus microplate reader (Tecan, Männedorf) in a light protected condition for 5 hours. Excitation and emission wavelengths used for fluorescence quantification were 485 nm and 535 nm respectively.

3.7. Molecular cloning and production of lentiviral particles

In this study we have used p47-roGFP, a redox biosensor that allows for noninvasive quantitative imaging of NADPH oxidase activity and pCOL1A1LV-tGFP, a human α(1) procollagen promoter to evaluate the activity of collagen synthesis in cells.

3.7.1. Cloning of the p47-roGFP biosensor construct

The coding sequences of human p47phox and roGFP2 were amplified by PCR from plasmid pcDNA3.1-p47-roGFP (kindly provided from Prof. George G. Rodney). XbaI and SalI restriction sites (underlined in primer sequence) were added to the forward (5'CGCTCTAGAATGGGGGACACCTTCATCCGT-3') and reverse (5'AGTCGACACTTACTTGTACAGCTCGTCCATG-3') primers, respectively. The PCR product was purified and cloned into the lentivector p156CMVGFP (kindly provided from Prof. Franco Galimi) using XbaI and Sal I restriction sites. The final construct has been checked by sequentiation.

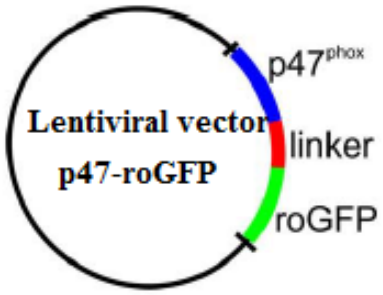


Figure 3.1. Construction of the Nox biosensor p47-roGFP.

3.7.2. Cloning of Human A(I) procollagen gene promoter

Human α(I) procollagen promoter (from -804 to +42) was cloned into a pJ241 plasmid using SalI and PmeI restriction sites. Then, the insert with Human α(I) procollagen promoter (from -804 to +42) was cut with SalI and PmeI restriction enzymes and subcloned into a lentivector containing tGFP protein. The final viral construct has been checked by sequentiation.

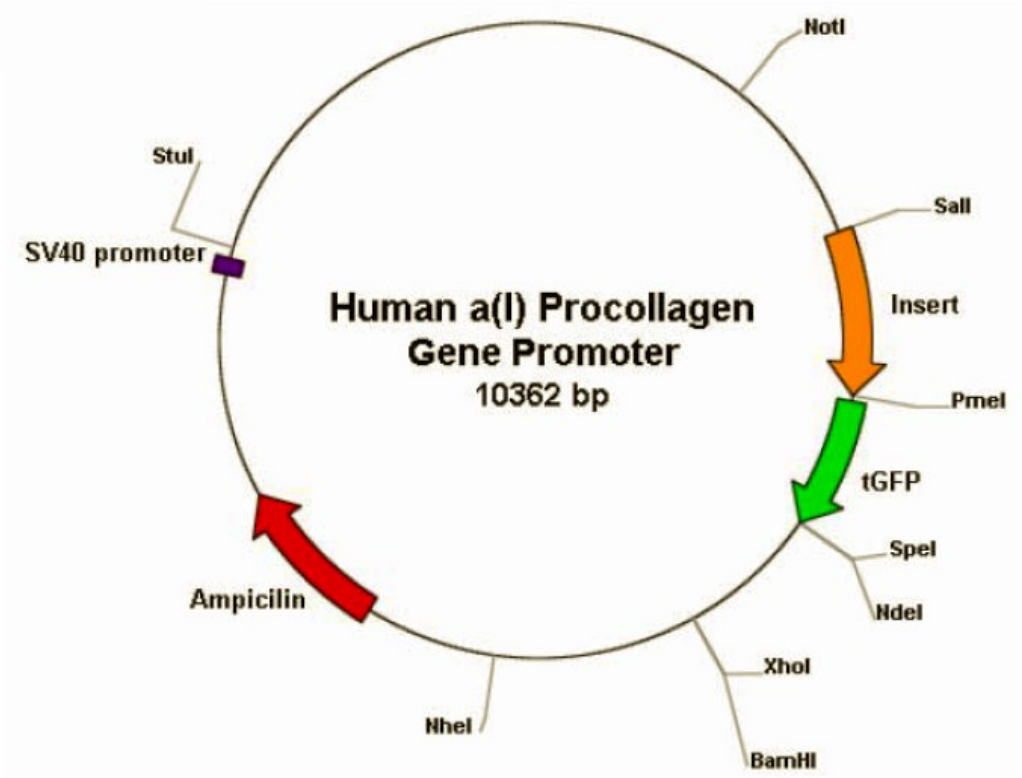


Figure 3.2. Human α(I) procollagen gene promoter-Lentivector-tGFP..

3.7.3. HPMEC transduction

Day0: In the T25 flasks, seed the HPMECs in Endothelial Cell Medium (ECM) (Innoprot, S.L, Spain) supplemented with 5% of FBS, 1% of ECGS, and 1% of P/S solution at appropriate density and incubate overnight (50%-75% confluent).

Day1: Remove the culture medium; add 1.5 mL of ECM complete medium to cover the surface of cell culture. Thaw the lentiviral stock at room temperature. Add appropriate amount of virus stock. Return cells to 37ºC/CO_2 incubator.

Day2: The day after, add ECM complete medium to reach 5 mL of volume.

Day3-4: At around 48-72h (depend on the protein) after transduction, check the protein production using fluorescence microscopy.

3.8. Determination of NOX2-associated ROS using NOX-specific redox biosensor p47-roGFP

Determination of NOX2-associated ROS was investigated by using the NOX-specific redox biosensor p47-redox-sensing green fluorescent protein (p47-roGFP). P47-roGFP, which was kindly provided from Prof. George G. Rodney, is a fusion of $p47^{phox}$, a subunit of NADPH oxidase 2 and roGFP2. P47-roGFP has two fluorescence excitation maxima at 400 (oxidized form) and 485 nm (reduced form) and display rapid and reversible ratiometric changes in fluorescence in response to changes in ambient redox potential. The ratios of fluorescence from excitation at 400 and 485 nm indicate the extent of oxidation and thus the redox potential while canceling out the amount of indicator and the absolute optical sensitivity. Fluorescence measurements were performed in black 96-well plates (Costar, Corning, Inc, NY) on a fluorescence microplate reader GENios plus (Tecan, Männedorf, CH). Cells were excited by using 400 and 485 nm filters and fluorescence values were measured using 535 nm emission filter. These values were averaged and subtracted from the fluorescence values of roGFP.

HPMECs transduced with p47-roGFP lentiviral particles (10^5 cells/mL) were seeded in black 96-well microplate (Costar, Corning, Inc, NY). Sub-confluent cells then cultured in basal medium containing 10% (V/V) of sera from SSc and HD subjects. Variations of redox status were kinetically followed for 5 hours and values at 2 hours were used for comparison.

3.9. Measurement of collagen promoter activity

Collagen promoter activity was investigated by employing pCOL1A1-LV-tGFP (Innoprot, S.L, Spain), a green fluorescence protein-based lentiviral vector transduced into the cells. This gene is driven by the human COL1A1

gene promoter. A red fluorescence was used to normalize the cell transduction efficiency. Fluorescence measurements were performed in black 96-well plates (Costar, Corning, Inc, NY) on a fluorescence plate reader GENios plus (Tecan, Männedorf, CH). Cells were excited by using 485 and 535 nm filters and fluorescence values were measured using 535 and 590 nm emission filter for pCOL1A1-LV-tGFP and EFα-LV-FP602, respectively.

HPMECs were transduced with lentiviral particles, which were obtained from the pCOL1A1-LV-tGFP and EFα-LV-FP602 lentivectors, and then cultured in basal medium containing 10% (V/V) of sera from SSc and HD subjects. Variations of COL1A promoter activation were kinetically followed for 48 hours. Data are normalized for transduction efficiency by reporting the ratio of pCOL1A1-LV-tGFP to EFα-LV-FP602 Relative Fluorescence Units (RFU).

3.10. Cell proliferation assay (BrdU incorporation Assay)

Cell proliferation was assessed by using chemiluminescent immunoassay, which based on the measurement of BrdU incorporation during DNA synthesis. When cells are pulsed with BrdU, it is incorporated into newly synthesized DNA strands of actively proliferating cells. The incorporation of BrdU into cellular DNA may then be detected using anti-BrdU antibodies, allowing assessment of the population of cells, which are synthesizing DNA.

The assay was performed in a black 96-well plate using a commercially available kit (ROCHE). $5x10^3$ cells per well were seeded in black 96-well plates and were cultured with M199 medium plus 2.5% FBS overnight. BrdU was added in each well of microplate, followed by incubation for 10 hours. Subsequently, supernatant was removed and was fixed by Fix-Denat solution for 30 minutes. Then, Fix-Denat was discarded and cells were incubated with an anti-BrdU antibody conjugated to peroxidase (anti-BrdU-POD) for 90

minutes. After rising three times with washing buffer, substrate solution was added and allowed to react for 3 minutes at room temperature. Finally, light emission was read by using a GENios plus microplate reader (Tecan, Mannedorf, CH). Results were expressed as mean ± SD.

3.11. Protein extraction

Cells were cultured in T12.5 culture flasks (BD Falcon). Sub-confluent HPMECs were treated with 10% (V/V) of sera of SSc patients and healthy donors for 72 hours. After 72-hour treatment, flasks were harvested and washed with chilled PBS. The cells were then incubated in ice-cold lysis buffer (CytoBuster protein extraction reagent; Novagen, Darmstadt, Germany) with freshly added protease inhibitor cocktails (Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktail 2, 3; Sigma, St Louis, MO) over ice for 10 min. The cell debris was scraped; the lysate was collected in a microfuge tube and cleared by centrifugation at 16,000g for 5 min at 4^0 C.

Supernatants were collected and stored in the same lysis buffer and stored at minus 80^0C. The protein content in the lysates was measured by Bradford assay following the manufacturer's protocol (Sigma, St Louis, MO).

3.12. Western Blotting

Protein extract and supernatant were subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE), and resolved proteins were transferred to a nitrocellulose membrane. The non-specific sites were blocked with blocking buffer (TBS containing 5% BSA and 0.1% Tween-20) for 2 hours on a rocker at room temperature. The blocked membrane was washed with washing buffer (TBS containing 0.1% Tween-20) three times (5 min) and then incubated with the appropriate primary antibody in blocking buffer at 4^0C overnight.

We used specific antibodies against von Willebrand factor protein vWF (Santa Cruz Biotechnology, INC), alpha-smooth muscle actin α-SMA (Novus Biologicals); and β-actin (Sigma-Aldrich, Saint-Louis, MO, USA).

The day after, the membrane was thoroughly washed with TBS/Tween, and incubated with a secondary antibody for one hour on the rocker at room temperature. Bands were detected using a peroxidase-conjugated IgG antibody and visualized by enhanced chemiluminescence. Beta actin was used as a loading control.

Protein content was determined by densitometric scanning of immunoreactive bands (Versadoc Imaging System; Bio-Rad). Results were expressed as arbitrary units, and ratios of individual densitometric results were normalized to β-actin immunoreactivity.

3.13. Statistical analysis

Data are shown for individual subjects in absolute values and presented as as mean ± SD. One or two way repeated measurement ANOVA followed by the Sidak's test was used to detect differences between more than two groups. Differences were considered to be statistically significant at $p < 0.05$. Statistical analysis was performed using GraphPad Prism 6.00 for Windows (GraphPad Software, San Diego, CA).

4. RESULTS

4.1. Clinical and serological characteristics of SSc patients.

SSc patients from Baltimore enrolled in the study were mainly middle age, white women. Subjects with SSc were slightly older than healthy donors (64.0 ± 9.4 vs 54.1 ±10.4; p = 0.0075).

Table 4.1. Clinical characterization of SSc patients in Baltimore

	SSc patients (n = 19)	Healthy donors (n = 14)	p value
Sex, female/male	16/3	12/2	
Age, median (range) years	64.0 ± 9.4	54.1 ± 10.4	0.0075
Race (white/black)	16/3	11/3	
Disease duration	21.7 ± 9.4		
SSc types (diffuse/limited)	3/16		
Autoantibodies			
Anti RNA polymerase 3	0		
Anti Scl -70	1		
Anti ACA	10		
Medication use (current)			
Immunosuppressants	5 (26%)		
Calcium channel blocker	7 (37%)		
Endothelin receptor antagonist	6 (32%)		
Phosphodiesterase 5 inhibitor	11 (58%)		
Prostanoid	0		
Statin	5 (26%)		
Aspirin	5 (26%)		

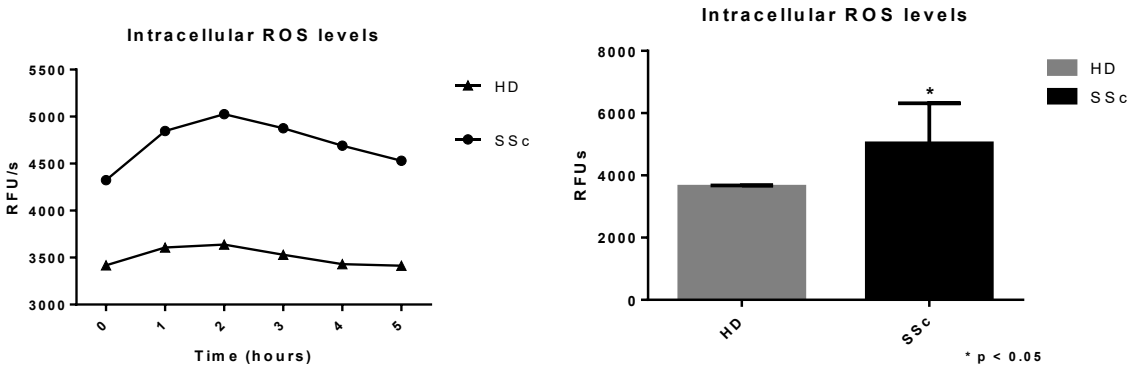
Similarly, SSc patients from Sassari enrolled in the study were mainly middle age, white women. Subjects with SSc were not significantly different compared healthy donors (p = 0.8203)

Table 4.2. Clinical characterization of SSc patients in Sassari

	SSc patients (n = 16)	Healthy donors (n = 14)	p value
Sex, female/male	12/4	12/2	
Age, median (range) years	55 ± 11	54.1 ± 10.4	0.8203
Race (white/black)	15/1	11/3	
Disease duration, mean ± SD	13 ± 12.3		
SSc types (diffuse/limited)	11/5		
Autoantibodies			
Anti RNA polymerase 3	1		
Anti Scl -70	10		
Anti ACA	2		
Medication use (current)			
Immunosuppressants	2 (12.5%)		
Calcium channel blocker	8 (50%)		
Endothelin receptor antagonist	2 (12.5%)		
Phosphodiesterase 5 inhibitor	2 (12.5%)		
Prostanoid	10 (62.5%)		
Statin	1 (6.25%)		
Aspirin	4 (25%)		

4.2. Sera of patients with SSc increased intracellular ROS level in HPMECs

We first tested whether sera of SSc patients could elicit a rise on intracellular ROS levels. Before stimulation, sub-confluent HPMECs were loaded with 10 µM H_2-DCFDA and then cultured in basal medium containing 10% (V/V) of sera from SSc patients and HD. Variations in intracellular ROS levels were kinetically determined in a 5-hour time-course experiment (Figure 4.1 A) and values at two hours, which show the maximum values, were used for comparison. As reported in the graphs, SSc sera significantly increased intracellular ROS levels in HPMECs.



(A) **(B)**

Figure 4.1. Effect of SSc sera on HPMEC production of intracellular ROS levels. Before stimulation, sub-confluent HPMECs were loaded with 10 µM H_2-DCFDA and then cultured in basal medium containing 10% (V/V) of sera from SSc patients and HD. Variations in intracellular ROS levels were kinetically determined in a 5-hour time-course experiment (Figure 4.1 A) and values at 2 hours were used for comparison (Figure 4.1 B). Fluorescence data were expressed as Relative Fluorescence Units (RFU). * $p < 0.05$: significantly different from control group (HD).

4.3. DPI reduced intracellular ROS levels induced by sera of SSc patient.

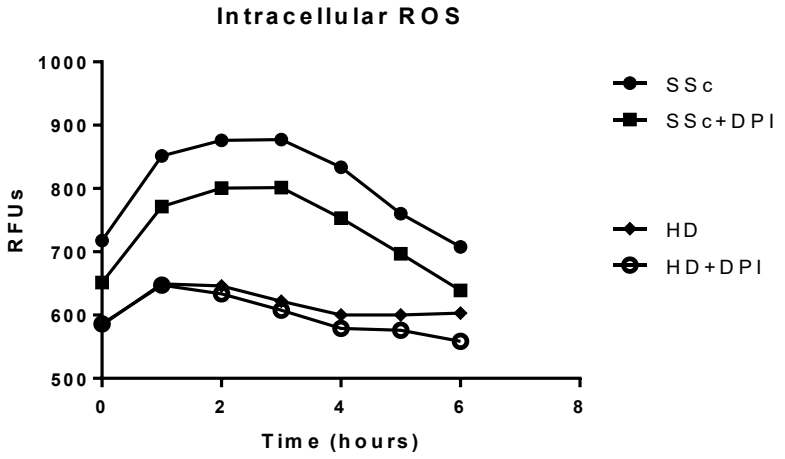


Figure 4.2. Diphenylene iodonium (DPI), a broad NOX inhibitor was exploited to investigate potential association between NOX family and the sera-induced ROS. HPMECs were treated with DPI 5μM for 1 hour, subsequently with 10% (V/V) of sera from SSc patients and HD. Variations in intracellular ROS levels were kinetically determined in a 6-hour time-course experiment. Fluorescence data were expressed as Relative Fluorescence Units (RFU).

Among the most important ligand-mediated sources of intracellular ROS are NADPH (NOX) oxidases. We therefore investigated the potential involvement of NADPH oxidases (NOX) in the increase of ROS elicited by sera of SSc patients. Diphenylene iodonium (DPI), a broad NOX inhibitor was exploited to investigate the association between NOX family and SSc sera-induced ROS generation. HPMECs were treated with DPI 5μM for 1 hour, subsequently cells were exposed to sera from SSc patients. After SSc sera exposition, HPMECs pretreated with DPI showed reduced levels of intracellular ROS as compared with cells that did not receive DPI, suggesting the implication of NOX in the ROS generation observed in SSc sera-exposed cells.

4.4. NOX2 is not involved in the increase of intracellular ROS levels induced by sera of patients with SSc.

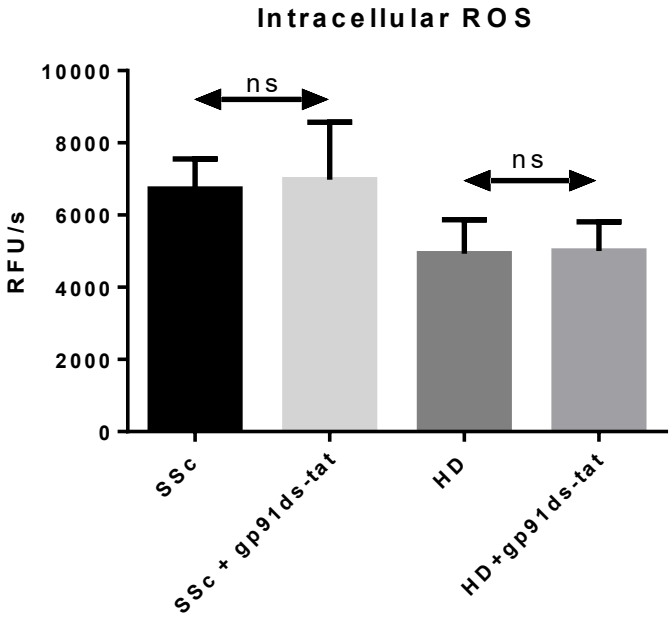


Figure 4.3. A specific NOX2 inhibitor (NOXds-tat or gp91ds-tat) was exploited to investigate whether NOX2 is related to NOX-derived ROS. HPMECs were treated with gp91ds-tat (NOX2 inhibitor) for 1 hour, subsequently with 10% (V/V) of sera of SSc patients and HD. Variations in intracellular ROS levels were kinetically determined in a 5-hour time-course experiment (not shown) and values at 2 hours were used for comparison. Fluorescence data were expressed as Relative Fluorescence Units (RFU). ns: non-significant difference.

As ROS-generating enzymes, seven NOX members are known so far, NOX1-5 and DUOX1-2. Previous result indicated that NOX2-mediated increase of ROS is induced by sera of SSc patients in HPASMCs [13]. NOX2, also known as gp91phox, is the prototype NADPH oxidase and a well-established player in the control of endothelial function. We therefore asked whether NOX2 might be implicated in the ROS generation observed under our experimental condition. To this end, we tested whether the specific NOX2 inhibitor (NOX2ds-tat or gp91ds-tat) was able to inhibit the increased levels

of intracellular ROS elicited by sera of SSc patients. HPMECs were pretreated with NOX2ds-tat (or gp91ds-tat) before the exposition to sera from SSc patients and the levels of intracellular ROS were measured. Data reported in figure 4.3 showed the failure of NOX2ds-tat in downregulating the increase of SSc sera-mediated ROS generation, questioning the implication of NOX2 in the observed oxidative stress.

To corroborate the above-reported result, we conducted a set of experiments using p47-roGFP. p47-roGFP is a fusion protein obtained by the binding of p47 (a subunit of NOX2) with the redox sensitive green fluorescence protein (roGFP), which is therefore able to assess environmental redox variation originated by NOX2 activation. Results reported in figure 4.4 show non-significant differences among SSc patients and HD in activating NOX2, thus, supports previous result and excludes NOX2 implication in SSc sera-mediated ROS production.

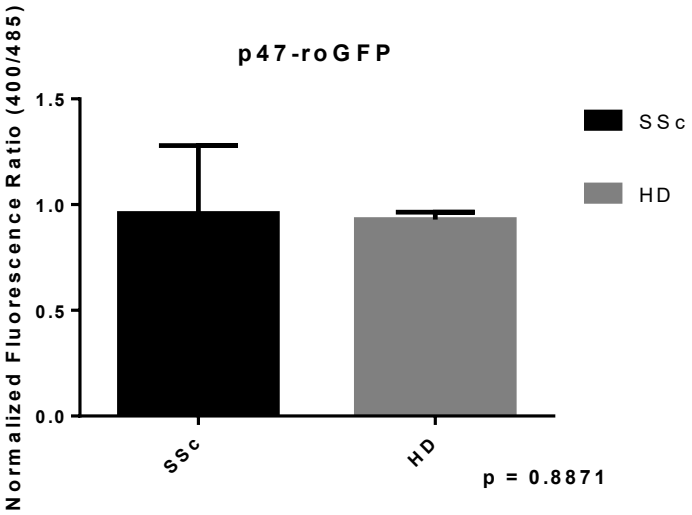


Figure 4.4. HPMECs transduced with p47-roGFP (10^5 cells/mL) were seeded in black 96-well microplate (Costar, Corning, Inc, NY). Sub-confluent cells were then cultured in basal medium containing 10% (V/V) of sera from SSc patients and HD. Variations of p47-roGFP redox status were kinetically followed for 5 hours and values at 2 hours were used for comparison.

4.5. Sera of patients with SSc raised COL1A1 promoter activity in HPMECs.

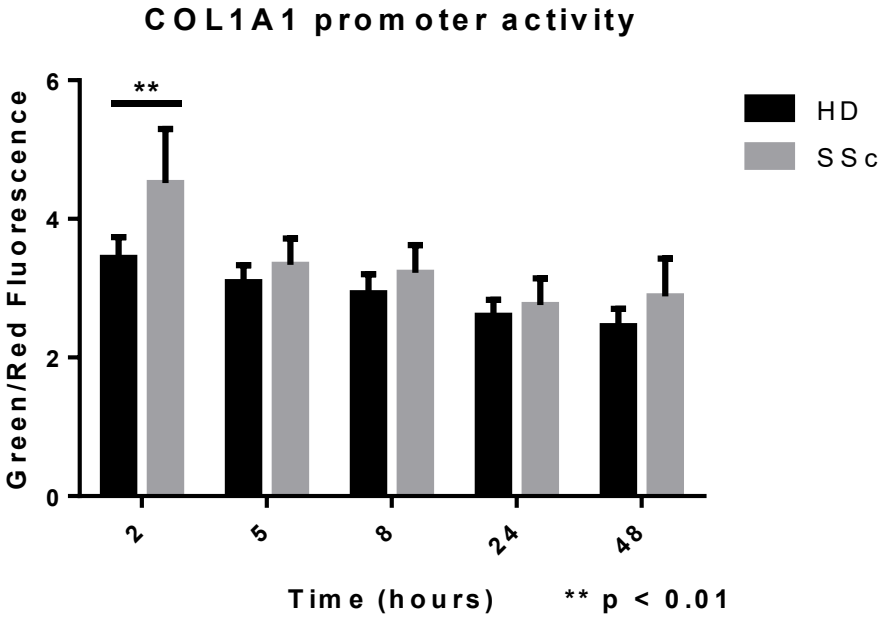


Figure 4.5. Effect of sera on collagen promoter activation. Sub-confluent HPMECs were transduced with lentiviral particles obtained from the pCOL1A1-LV-tGFP and EFα-LV-FP602 lentivectors, and then cultured in basal medium containing 10% (V/V) of sera from SSc and HD subjects. Variations of COL1A promoter activation were kinetically followed for 48 hours. Data are normalized for transduction efficiency by reporting the ratio of pCOL1A1-LV-tGFP to EFα-LV-FP602 Relative Fluorescence Units (RFU) (Green/Red fluorescence). Results are expressed by mean ± SD. ** $p < 0.01$: significantly different from control group (HD).

Collagen type I is a key player in both the onset and progression of many obliterative vascular diseases including SSc. Therefore, we next wanted to determine whether SSc sera possess the capability to increase type I collagen production in HPMECs. Collagen type I synthesis was investigated by infecting the cells with pCOL1A1-LV-tGFP, a turbo green fluorescence protein (tGFP)-based lentiviral vector, which is driven by the human COL1A1 gene promoter.

Data shown in figure 4.5 indicate that sera of SSc patients significantly increased collagen promoter activation during the first 2 hours of exposition, thereafter the COL1A1 promoter activity decreased, and remaining stable for the entire experimental time-course (48 hours).

4.6. Sera of patients with SSc enhanced proliferative capacity of HPMECs.

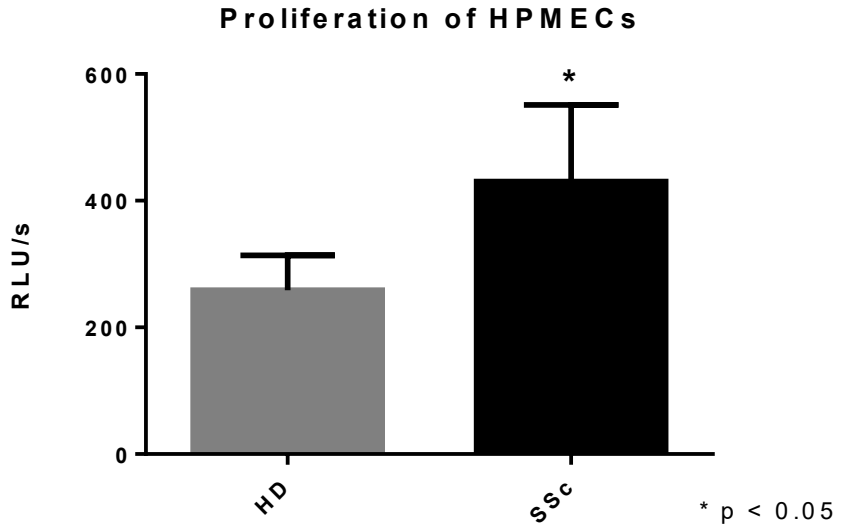
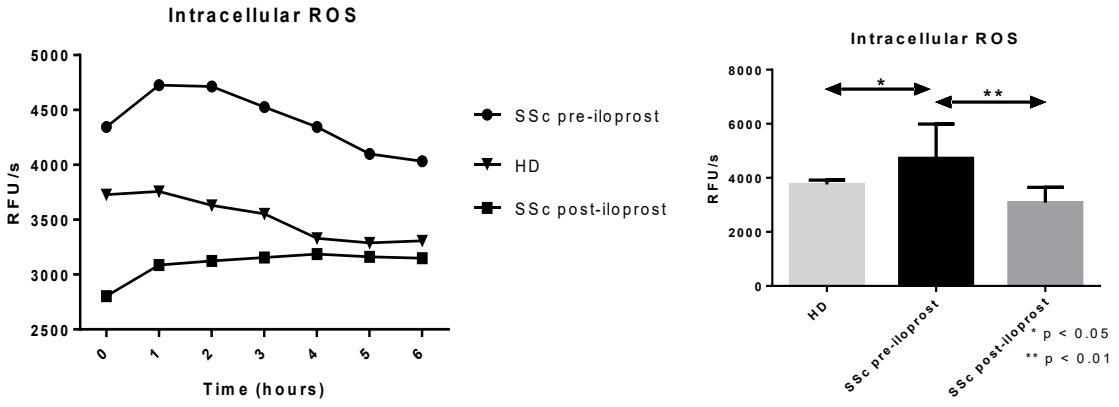


Figure 4.6. Effect of sera from SSc patients on HPMEC proliferative capacity. HPMECs were starved overnight then treated with sera of SSc patients and HD for 24 hours. Results are expressed by mean ± SD. * $p < 0.05$: significantly different from control group (HD).

Based on their concentration, ROS can be important modulator of both physiological and pathological cell proliferation. To understand whether the variations of intracellular ROS may affect HPMEC proliferation, we assessed the rate of DNA synthesis in cells exposed to SSc and HD sera. The method employed is based on the measurement of BrdU incorporation during DNA synthesis. HPMECs were starved overnight then treated with sera of SSc patients and HD for 24 hours. In comparison to the sera from HD, SSc sera originated a significantly higher proliferative effect on HPMECs ($p < 0.05$).

4.7. Iloprost significantly reduced intracellular ROS levels in HPMECs

Given the implication of ROS in the SSc sera-elicited effects on HPMECs, we asked whether iloprost, a common drug used in the treatment of SSc, might exert its therapeutic benefits through an antioxidant effect. As stated previously in chapter 3 (Materials and Methods), sera were collected at two different time points, the first sampling at the baseline and the second sampling after 5 hours of the iloprost intravenous infusion. Intracellular ROS levels were kinetically determined in a 6-hour time-course and values at the first hour were used for comparison as they represented the higher values of the time-course. As reported in figure 4.7, the sera of SSc patients taken before iloprost infusion significantly increased ROS levels in HPMECs, while sera of SSc patients taken 5 hours after the iloprost infusion significantly countered the diseases-associated ROS generation ($p < 0.01$).



(A) **(B)**

Figure 4.7. Intracellular ROS levels induced by sera of patients with SSc before and after using iloprost. Sub-confluent HPMECs were stimulated with sera of SSc before and after using iloprost (SSc pre-iloprost and SSc post-iloprost, respectively). Intracellular ROS levels were kinetically determined in a 6-hour time-course (A) and values at first hour were used for comparison (B). The results are expressed with mean ± SD. *$p < 0.05$; ** $p < 0.01$: significantly different from control group (HD).

4.8. Iloprost reduced COL1A1 promoter activity in HPMECs

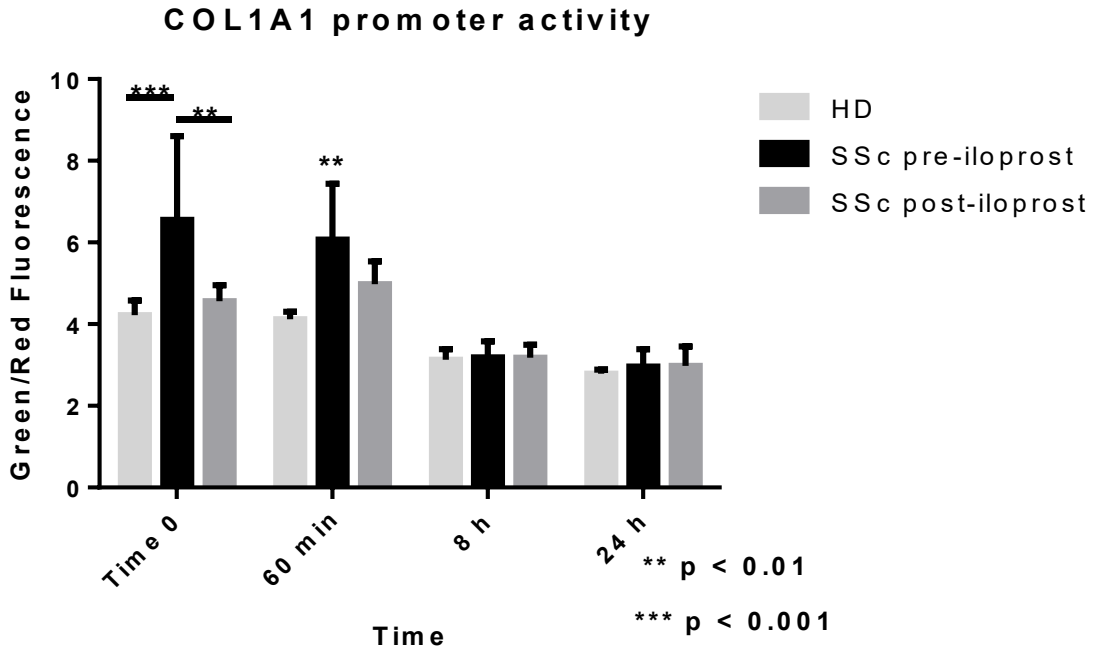


Figure 4.8. Effect of SSc sera taken at the baseline and after 5 hours using iloprost on collagen promoter activation. Sub-confluent HPMECs were transduced with lentiviral particles obtained from the pCOL1A1-LV-tGFP and EFα-LV-FP602 lentivectors, and then cultured in basal medium containing 10% (V/V) of sera from SSc (before and after using iloprost), and HD subjects. Variations of COL1A promoter activation were kinetically followed for 24 hours. Data are normalized for transduction efficiency by reporting the ratio of pCOL1A1-LV-tGFP to EFα-LV-FP602 Relative Fluorescence Units (RFU) (Green/Red fluorescence). Results are expressed by mean ± SD. ** $p < 0.01$; *** $p < 0.001$: significantly different from control group (HD).

Given the ability of iloprost in counteracting the disease-associated variation of ROS, we asked whether this affect may be paralleled with the inhibition of SSc-induced collagen I activation. To this end, the variations of COL1A promoter activation were kinetically measured for 24 hours. The result shows

type I collagen promoter activity prominently occurred in the first hour. SSs serum-induced activation of collagen synthesis was significantly higher compared to control group. Interestingly, SSc sera collected after using iloprost reduced the collagen synthesis, suggesting this phenomenon as potential target of the iloprost therapeutic effect.

4.9. Sera of SSc patients induces Endothelial-to-Mesenchymal Transition

Disease-associated vascular damage is usually associated to endothelial impairment, which is characterized by endothelial cells (ECs) with less proliferative potential and lower angiogenic capability [3]. Surprising, as reported in figure 4.6, sera from SSc patients were instead able to increase HPMECs proliferation. In this context, the capability of ECs to shift their cellular phenotype toward a more fibrotic and proliferation one, namely myofibroblast, has been reported [64]. During this transition, called Endothelial-to-Mesenchymal Transition (EndMT), the EC leaves its physiological state of quiescence, becoming more proliferative and acquiring high capability of migration and secreting extracellular matrix components [64]. To test whether SSc sera are able to induce the conversion of ECs into myofibroblasts, HPMECs were exposed to sera of SSc patients and HD for 72 hours, and endothelial and fibrotic characteristics were measured by using two specific markers of both phenotypes. ECs exposed to SSc sera exhibited a decrease in the protein levels of the endothelial characteristic marker - vWF (Figure 4.10). Furthermore, consistent with the establishment of a fibrotic process, ECs challenged with SSc sera showed an increase in the fibrotic markers, α-SMA (Figure 4.9).

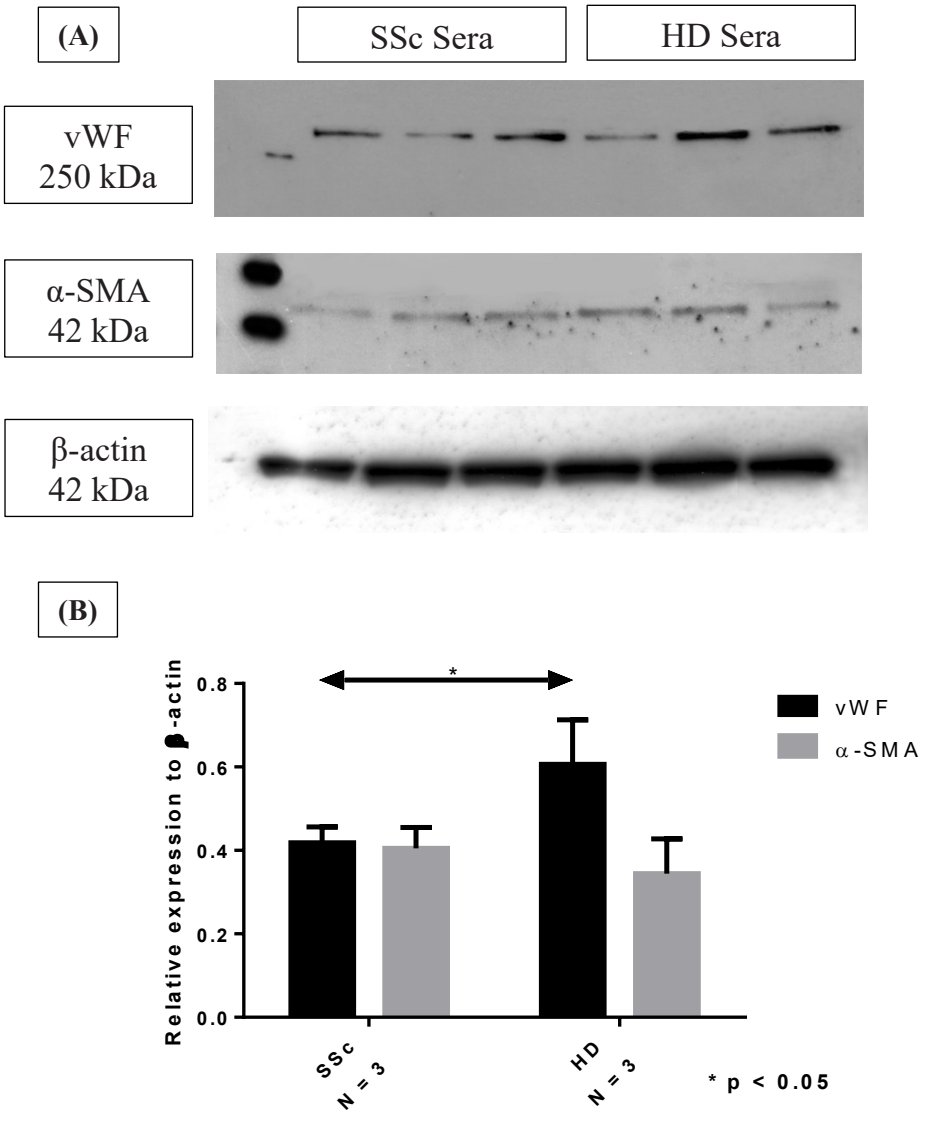


Figure 4.9. Analysis of endothelial and mesenchymal cell surface markers by Western blot analysis. Sub-confluent cells were stimulated with sera of SSc patients and HD for 72 hours and then processed for immunoblotting as described in the "Materials and Methods". α-SMA, α-smooth muscle actin; vWF, von Willebrand factor.

5. DISCUSSION

Recently, many reports underline a possible role of oxidative stress in the pathogenesis of SSc. The production of reactive oxygen species (ROS) by skin, visceral fibroblasts, and endothelial cells has been suggested as a background of this pathology [51, 94, 105]. SSc-associated oxidative stress causes both activation and endothelium damage, ultimately leading to vascular complications [51]. Consonantly, *in vitro* exposure of fibroblast to sera from SSc patients induces high production of ROS, whose levels are correlated with the severity of clinical involvement [98, 109]. As ROS can modulate cell activation, and since endothelial cells are the primary target in SSc, endothelial dysfunction may be the first pathological event in the pathogenesis of SSc-associated vascular disease. By testing the above-mentioned hypothesis, we demonstrated for the first time that sera from SSc patients induce an abnormal ROS production in HPMECs.

Within the cells, ROS can be generated from different sources including the mitochondrial electron transport chain, xanthine oxidase, cyclooxygenase, and lipoxygenase. Nonetheless, among all the cellular sources, NADPH oxidases are the most important mediators of ligand-mediated ROS production. NADPH oxidases (NOX) are multisubunit enzymes that generate superoxide by transferring electrons from NADPH to molecular oxygen [50]. In phagocytes, ROS originating from NOX participate in bacterial killing, whereas in nonphagocytic cells they are required for cellular responses to cytokines and growth factors [72]. To investigate whether the observed sera-induced ROS derive from NOX family, we conducted an experiment employing a general NOX inhibitor, diphenylene iodonium (DPI). HPMECs were treated with DPI for one hour prior to the treatment of sera from SSc patients. The results showed that DPI, potentially by inhibiting NOX, reduced

ROS levels in HPMECs (figure 4.2), therefore suggesting an involvement of NOX in ROS generation of HPMECs.

Endothelial cells express four NOX isoforms including the superoxide-generating enzymes NOX1, NOX2, NOX5 and the hydrogen peroxide-generating enzyme NOX4 [32]. The three most important NOX isoforms related to SSc are NOX1, NOX2, and NOX4 [96]. NOX2 was the first NOX isoform to be identified in endothelial cells and, it is likely to be the most important in the context of vascular pathology**.** We therefore asked whether NOX2 might be implicated in the ROS generation observed under our experimental condition. To this end, we tested whether the specific NOX2 inhibitor (NOX2ds-tat or gp91ds-tat) was able to inhibit the increased levels of intracellular ROS elicited by sera of SSc patients. HPMECs were pretreated with NOX2ds-tat (or gp91ds-tat) before the exposition to sera from SSc patients and the levels of intracellular ROS were measured. Experiment exploiting NOX2 inhibitor did not show any difference between disease- and non-disease-related sera. This may indicate that NOX2 is not implicated in SSs-elicited ROS increase. In the NOX2, p47phox is a subunit whose phosphorylation leads to its movements toward other NOX2 cytoplasmic subunits to form the active enzyme complex of NOX2. The active enzyme complex works by generating superoxide ($O_2^{\cdot -}$) in response to the ligand-mediated activation. Notably, p47 exists only in NOX2; hence, we conducted a set of experiment using p47-roGFP, a fusion of p47 and redox green fluorescence protein to investigate NOX2-generating ROS. Similar to the previous reported data, SSc sera were unable to induce significant variation of p47-roGFP as compared with HD sera, indicating that NOX2 may not be involved in SSc serum-induced ROS. However, in the study of Boin *et al*., NOX2 inhibitor (NOX2ds-tat) pretreatment in HPASMCs inhibited ROS

production induced by sera from SSc patients [13]. Although NOX2 is likely the most important in the context of vascular pathology, NOX4 is the most highly expressed NOX homolog in endothelial cells. Recent evidence indicating that the NOX4 may be one of the most important mediators of ROS generation in SSc and the potentially beneficial effects of inhibition of NOX4 activity is a cogent therapeutic approach for SSc-associated tissue fibrosis and fibroproliferative vasculopathy [62, 96]. Probably NOX4 plays the prominent role in ROS generation in this circumstance and will be therefore the object of our future investigation.

A hallmark characteristic of SSc that distinguishes it from other fibrotic disorders is that autoimmunity and vasculopathy precede fibrosis. Moreover, fibrosis in SSc affects many organs and accounts for much of the morbidity and mortality related to this disease [121]. The progressive tissue accumulation of fibrous matrix composes of collagen, elastin, glycosaminoglycan, and fibronectin [2]. Fibrosis may be exacerbated by the failure of intrinsic mechanisms that normally constrain fibroblast activation [35]. The study of Boin *et al.* demonstrated that sera of SSc patients increased COL1A1 promoter activity in HPASMCs [13]. Interestingly, our study also supports the hypothesis that serum of SSc patient is a contributory factor in increasing collagen synthesis leading to progressive fibrosis in this disease. Aside from fibroblasts, the main determinant accounting for the collagen synthesis and fibrosis in SSc are smooth muscle cells and endothelial cells, which are less-mentioned sources of collagen synthesis in the pathogenesis of fibrosis in SSc. A need of further studies on this issue urges at moment, in order to be able to reverse or slow the progression of tissue fibrosis or substantially modify the natural progression of the disease.

A study described a novel mechanism by which ROS may promote a profibrotic phenotype in SSc fibroblasts. ROS production and expression of type I collagen, a fibrotic marker gene, were significantly higher in SSc, and these alterations were accompanied by significantly lower amounts of free thiols compared to normal fibroblasts. Treatment of SSc cells with antioxidant decreased the amounts of type I collagen, and scavenged ROS in SSc fibroblasts [119]. Likewise, our results indicated that SSc sera induce increased ROS generation and collagen promoter activity. Increase in ROS levels in HPMECs may cause the elevated activation of collagen promoter. Consistently with other studies, our work collectively provides strong support for the participation of ROS in the development of exaggerated fibrotic responses in SSc.

At physiological levels, cellular ROS control cell growth and differentiation regulating the activity of enzymes, mediating inflammation, stimulating cytokine production and eliminating pathogens and foreign particles. On the other hand, if present in excessive levels, ROS cause the damage of cellular components, including lipids and membranes, proteins and nucleic acids, and ultimately inducing cell transformation. Unexpectedly, rather than eliciting cell death, the observed SSc-ROS generation was able to accentuate it. SSc is a fibro-proliferative disease characterized by the abnormal proliferation and extracellular matrix secretion of most of the involved cells [60]. Nevertheless, the above-mentioned aspect, namely Endothelial-to-Mesenchymal Transition (EndMT) in the case of ECs, has never been investigated a molecular level in SSc disease.

It was hypothesized that endothelial cells undergoing EndMT may contribute to endothelial dysfunction and the population of activated mesenchymal cells in fibrotic and chronic inflammatory disorders, including inflammatory bowel

disease, cardiac fibrosis, and portal hypertension [48]. In the pathogenesis of SSc, one of the most characteristic histopathologic alterations is a severe fibroproliferative vasculopathy. The alteration in the small arterioles of parenchymal organs, such as the lungs and the kidneys, is characterized by the subendothelial accumulation of activated fibroblasts or myofibroblasts and the production of abundant fibrotic tissue. The origin of mesenchymal cells responsible for the fibrotic microvascular occlusion in SSc is not known, but recent studies have suggested that at least some of these cells may result from EndMT [64]. Under the conversion of endothelium into mesenchyme, endothelial cells undergo morphological alterations and loss of cell-surface markers. Our result for the first time indicated the presence of EndMT in normal HPMECs exposed to sera of SSc patients. Indeed, the expression of vWF, a specific endothelial cell marker, was gradually replaced by α-SMA, a mesenchymal or myofibroblastic marker. The acquisition of α-SMA is a prominent functional feature of myofibroblasts that changes endothelial functionality. Phenotypic transformation of ECs leading to decreased levels of endothelial adhesion proteins, which allow cell-to-cell separation and generate the spindle-shaped, fibroblast-like phenotype, which is strongly potentiated by the overexpression of α-SMA stress fibers [88]. Loss of an integral endothelial barrier is believed to contribute to the activation and proliferation of the underlying vascular SMCs and fibroblasts, thus contributing to vascular remodeling [48]. This finding contributes to the literature of etiological mechanism of SSc-associated vascular damage, as well as suggests a potential therapeutic target to inhibit obliterative vascular disorder and tissue fibrosis.

Iloprost, an analogue of natural prostacyclin (PgI2), is widely used in SSc for the treatment of severe RP and ischemic ulcers due to its powerful vasodilator

and anti-aggregant effect. Iloprost is effective in the treatment of RP secondary to SSc at decreasing the frequency and severity of attacks and preventing or healing digital ulcers [36, 122]. There is evidence that iloprost attenuates oxidative damage induced by ischemia-reperfusion phenomenon. Ferrari *et al.* demonstrated that pretreatment with iloprost improved the functional recovery of the myocardium and attenuated the occurrence of oxidative stress on rabbit isolated hearts. Similarly, other studies have showed that iloprost reduced oxidative stress in ischemia-reperfusion model of skeletal muscle, brain, and renal tissue [122].

Although some studies investigating the effect of iloprost on oxidative stress and collagen synthesis have been published, very little is known concerning the molecular mechanism underlying this effect. To our knowledge, this is the first study employing the serum of SSc patient taken after treatment with intravenous iloprost and investigates its effect on pulmonary endothelial cells. Our results showed that SSc sera post-iloprost significantly reduced ROS levels in HPMECs (figure 4.7). A study of Erre *et al.* demonstrated that a cycle of five days of iloprost infusion at standard dosage leads to a significant reduction of 8-iso prostaglandin-$F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) urinary levels in the same SSc patients. As noted, 8-iso $PGF_{2\alpha}$ is a biochemically stable member of the family of F2-isoprostanes, free radical catalyzed products of lipid peroxidation that are considered the best reliable markers of oxidative stress *in vivo* [36]. Apparently, recent studies indicate that dietary and pharmacological antioxidants can be used as therapeutic targets for SSc [51]. This study thereby supports the evidence that iloprost could be used as antioxidant therapy aside from its vasodilatory effect in SSc patients.

Scleroderma patients receiving iloprost as a treatment for severe Raynaud's phenomenon report reduction in skin tightness, suggesting that this drug

inhibits skin fibrosis [116]. Our results showed that HPMECs treated with sera after iloprost infusion markedly reduce type I collagen synthesis.

Stratton *et al.* demonstrated that iloprost infusion caused a remarkable decrease in dermal CTGF levels. CTGF is a well-known profibrotic cytokine, which acts in concert with TGF-β to drive the overproduction of collagen [15]. Consistent with this, Gomez-Arroyo *et al.* also indicated *in vitro*, cardiac fibroblasts treated with iloprost showed a reduction in TGF-β1-induced CTGF expression. Iloprost significantly induced metalloproteinase-9 gene expression and activity and increased the expression of autophagy genes associated with collagen degradation [47]. In our stydy, iloprost infusion in SSc patients might attenuate CTGF levels in SSc sera preceding the alleviation of type I collagen synthesis. Indeed, in SSc, skin and visceral fibroblasts spontaneously produce large amounts of ROS that trigger collagen synthesis [51]. Therefore, the decrease in ROS levels induced by post-iloprost sera infusion as shown in previous result may explain the reduction of type I collagen synthesis in pulmonary endothelial cells.

We also found that sera after being treated iloporost suppressed type I collagen synthesis at the first 2 hours after treatment and that this effect was lost by 8 hours (24 hour-time course mesurement). These observations have implications for the clinical use of iloprost, suggesting that a brief exposure to the drug two or three times a day might be sufficient to maintain suppression of collagen promoter activity. The results of the present study suggest that iloprost infusion could have beneficial effects in addition to its properties as a vasodilator.

Although this work provides some findings concerning the etiology of SSc-associated vascular damage, at the same time it opens new questions and challenges. For instance, whether SSc sera induced-oxidative stress is the

mediator of EndMT in SSc and iloprost may counteract such a phenomenon remains unelucidated. Further investigation needs to be conducted to discover the causative factors leading to the conversion of ECs into myofibroblasts in SSc. Therefore, whether there is a link between oxidative stress and EndMT in SSc is an intriguing question, which requires more investigations to answer.

CONCLUSION

With the intent to investigate the molecular mechanisms underlying vascular damage in systemic sclerosis (SSc), we achieved some pilot results.

For the first time we report that SSc sera significantly increased ROS levels, proliferation, and collagen synthesis in human pulmonary microvascular endothelial cells, as compared with sera from Healthy Donor (HD). The observed effects appear to be mediated by NADPH oxidases suggesting the potential for an antioxidant therapeutic approach. Interestingly, SSc sera taken after 5 hours of iloprost infusion could attenuate ROS levels and collagen synthesis. Thus, our study suggests that, aside from its well-known vasodilatory effect, an antioxidant-mediated effect could be part of the mechanisms of action of this drug. Sera from SSc patients also induced EndMT indicating this phenotypic shift as an important etiological mechanism of SSc-associated vascular damage, as well as a potential therapeutic target to inhibit obliterative vascular disorder and tissue fibrosis.

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