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Role of HLA-B35 and ER stress in Scleroderma patients with Pulmonary Arterial Hypertension

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TABLE OF CONTENTS

List of Figures

Abstract

Pulmonary arterial hypertension (PAH), a common complication of limited cutaneous systemic sclerosis (lcSSc), is associated with alterations of markers of inflammation and vascular damage. Endoplasmic reticulum (ER) stress and unfolded protein response (UPR) have been implicated in various diseases. The presence of the HLA-B35 allele, Human antigen class I, has emerged as an important risk factor for the development of PAH in patients with lcSSc, however the mechanisms underlying this association have not been fully elucidated.

We have recently reported that the presence of HLA-B35 contributes to human dermal microvascular endothelial cell (HDMEC) dysfunction by significantly increasing production of endothelin-1 (ET-1) and significantly decreasing endothelial NO synthase (eNOS). Furthermore, HLA-B35 greatly upregulated expression of chaperones, including heat shock proteins (HSPs) HSP70 (HSPA1A and HSPA1B) and HSP40 (DNAJB1 and DNAJB9), suggesting that HLA-B35 induces the ER stress and UPR in ECs and this mechanism can mediate the induction of ET-1 in patients with PAH.

The goal of this study was to better understand the role of HLA-B35-induced ER stress/UPR in the development/progression of PAH disease in lcSSc patients.

First we focused on the molecular mechanisms of ET-1 induction by HLA-B35. ER stress inducer, Thapsigargin (TG) and HLA-B35 induced ET-1 expression with similar potency in HDMECs. HLA-B35 or ER stress activated the PERK/eIF2α/ATF4 branch of the UPR and modestly increased the spliced variant of X-box binding protein (XBP1), but did not affect the Activating Transcription Factor -6 (ATF6) pathways.

Depletion of ATF4 decreased basal expression levels of ET-1 mRNA and protein, and completely prevented upregulation of ET-1 by HLA-B35/ER stress. Additional experiments have demonstrated that the JNK and NF-κB pathways are also required for ET-1 upregulation by HLA-B35/ER stress. Formation of the ATF4/c-JUN complex, but not the ATF4/NF-κB complex was also increased. The functional role of c-JUN in responses to HLA-B35/ER stress was further confirmed in ET-1 promoter assays. This study identified ATF4 as a novel activator of the ET-1 gene.

Then we focus on whether markers of ER stress/UPR were present in PBMCs from lcSSc-PAH patients and if the presence of HLA-B35 contributes to activation of the immune cells. Several ER stress/UPR genes, including Immunoglobulin-heavy-chain binding protein (BiP), ATF4 and ATF6 and a spliced form of XBP1 were upregulated in lcSSc PBMCs, with the highest levels in patients with PAH. Also selected HSP genes, particularly DNAJB1, and IFN-related genes were found at significantly elevated levels in PBMCs from lcSSc patients, while IRF4 was significantly decreased. There was a positive correlation between DNAJB1 and severity of PAH disease (PAP) ($r = 0.56$, $p<0.05$) and between ER stress markers and IL-6 levels ($r = 0.53$, $p< 0.0001$) in lcSSc PBMCs.

When we stratified all PBMC samples based on the presence of the HLA-B35 allele, we could observe that HLA-B35 positive individuals showed higher levels of selected ER stress markers when compared to HLA-B35 negative individuals. Furthermore, patients carrying HLA-B35 antigen expressed higher levels of IL-6, a key inflammatory cytokine associated with development of PAH. This study demonstrates association between select ER stress/UPR markers and lcSSc-PAH suggesting that ER

stress/UPR may contribute to the altered function of circulating immune cells in lcSSc. All these associations were enhanced by the presence of HLA-B35.

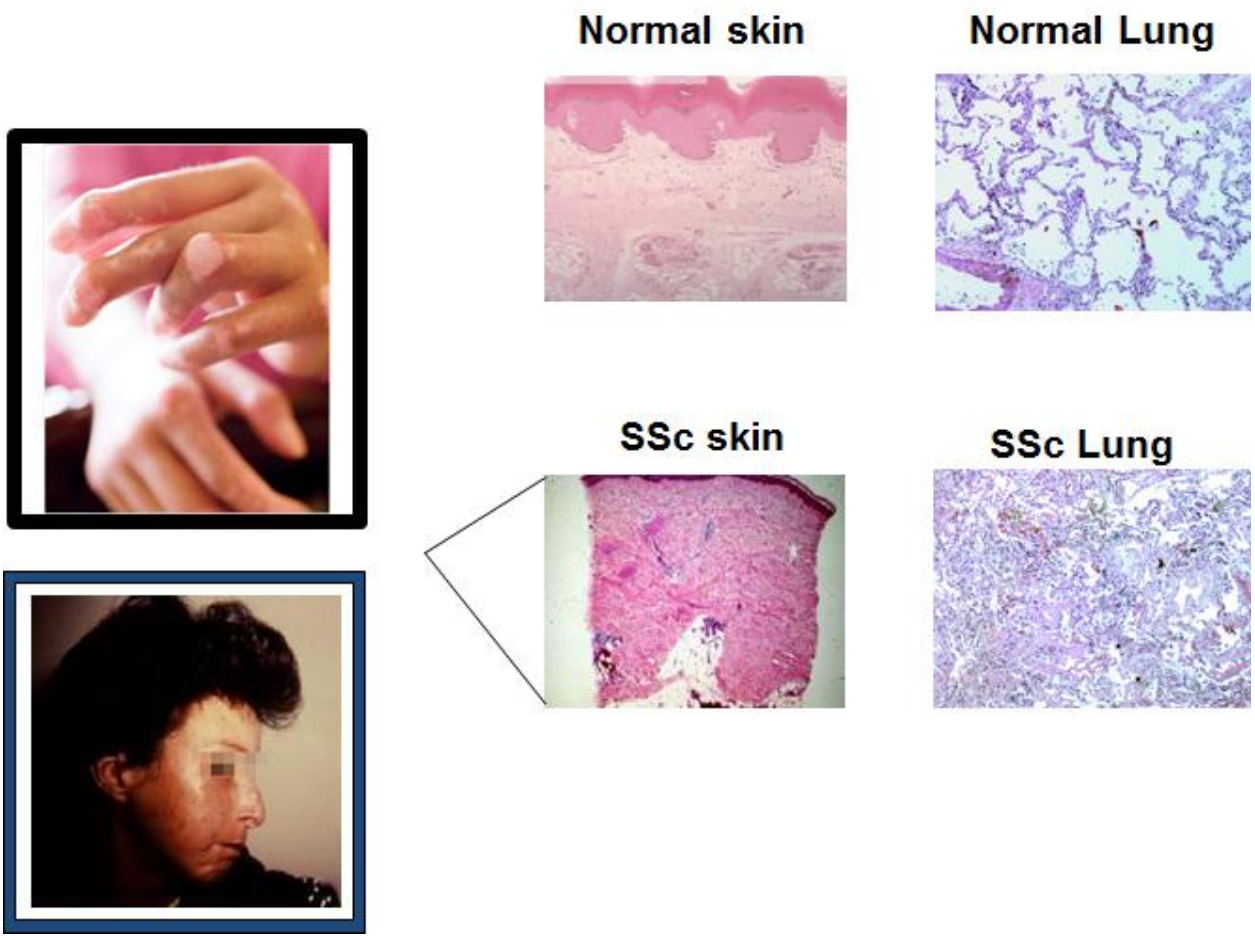
In conclusion, we hypothesize that HLA-B35 may play a role in EC dysfunction inducing ET1 via ER stress/UPR. Also activation of ER stress/UPR, in combination with presence of HLA-B35, might drive the inflammatory process in lcSSc-PAH.

Chapter 1

Introduction

Scleroderma

Scleroderma or Systemic Sclerosis (SSc), is a complex autoimmune disease of unknown origin characterized by extensive pathological ECM remodeling resulting in fibrosis of the skin and internal organs (1). SSc is a highly heterogeneous disease, with manifestations from limited skin involvement to diffuse skin fibrosis and extensive internal organ involvement.(2) SSc is characterized by excessive deposition of collagen and other extracellular matrix proteins in the skin and multiple internal organs, degeneration of the microvasculature, and abnormalities in the cellular and humoral immune system. Morphological changes in the skin include deterioration of the hair follicles and sebaceous glands, dramatic dermal thickening, epidermal degeneration, and overall tightening (3). In early lesions, massive leukocyte infiltration is observed at the border between epidermis and subcutaneous fat layers (4). The skin of the face and hands is most frequently affected and skin ulcerations are common (3). Internal organ fibrosis in SSc is most frequently incurred in the gastrointestinal tract but pulmonary, renal, and cardiac involvement lead to the most serious and possibly fatal symptoms .The mortality rate for systemic SSc is about 55% at 10 years (5-7). No cure has been found for SSc and current treatments are ineffective.



Fugure 1.1. Scleroderma features.

Scleroderma is characterized by thickening, tightening, and induration of the skin of the fingers .These changes may affect the entire extremity, face, neck, and trunk.

Possible causes of SSc

Although the etiology of SSc is unknown, several possible hypotheses of the etiological agent of SSc pathogenesis have been proposed. Exposure to various environmental stressed (toxins and chemicals) has been implicated in developing SSc (7-9). A genetic component is supported by familial clustering patterns and high prevalence of the disease among certain ethnic groups (1). Various ethnic populations of SSc patients exhibit specific expression patterns of human leukocyte antigens (HLAs), major histocompatibility complex (MHC) alleles, and autoantibodies (3). Several single nucleotide polymorphisms (SNPs) are associated with SSc in genes encoding for vasomotor regulatory factors, B-cell markers, chemokines, chemokine receptors, cytokines, growth factors and their receptors, connective tissue growth factor (CCN2), transforming growth factor β (TGF-β), and extracellular matrix (ECM) proteins (1). Infection of herpes virus, retrovirus, and human cytomegalovirus have been proposed as possible etiological agents in SSc development (3, 9-11)

In SSc there are three main features involved: vascular damage (complete obliteration of microvessels), production of autoantibodies (by B and T cells) and activation of fibroblasts (production of excess extracellular matrix) (1). Although the initial mechanisms of the disease are not fully understood, it is suggested that endothelial cell injury stimulates an immune response with B and T cell activation and subsequent release of pro-inflammatory mediators, growth factors, and cytokines [12]. One of the major growth factors secreted by the immune cells is transforming growth

factor-β (TGF-β), which is important in wound-healing processes as well as plays a key role in the process of fibrosis.

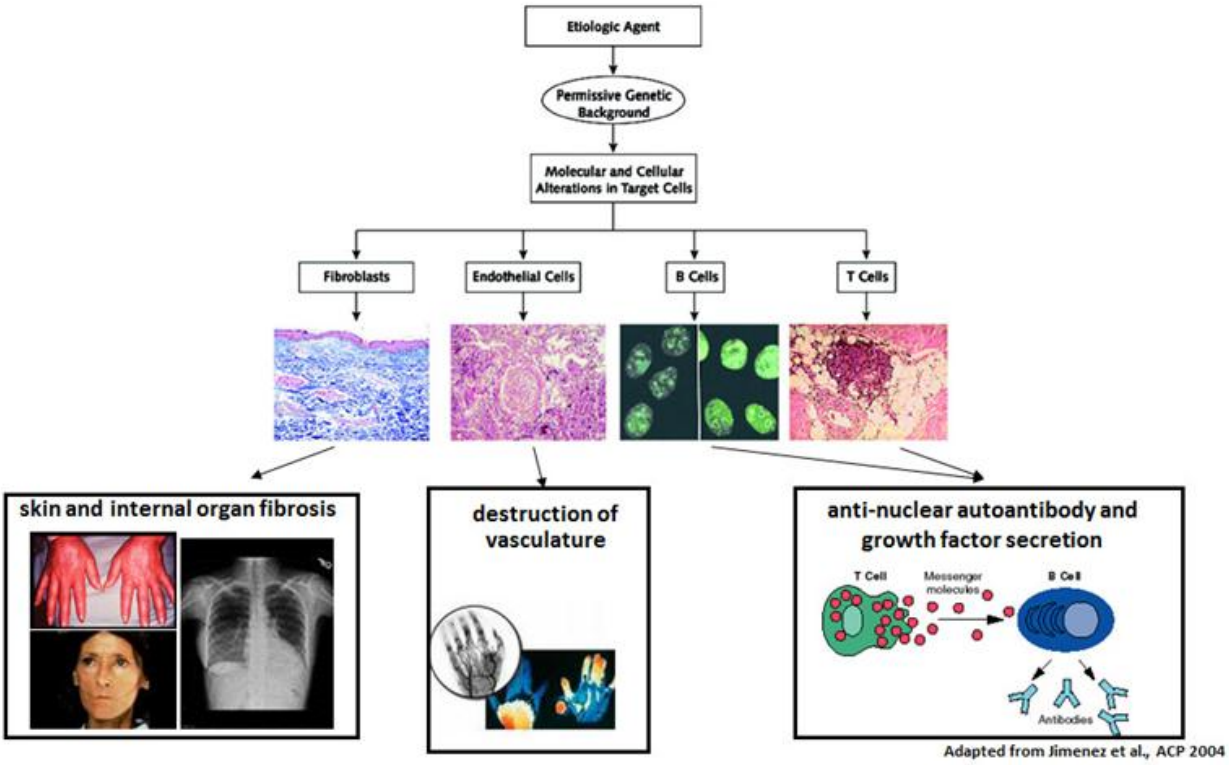


Figure 1.2. Abnormalities in 3 systems: ECM, Immune, and Vascular.

In SSc there are three main features involved: vascular damage (complete obliteration of microvessels), production of autoantibodies (by B and T cells) and activation of fibroblasts (production of excess extracellular matrix.

Fibrosis

SSc fibroblast activation to produce ECM proteins occurs as a result of growth factors released from immune cells into the microenvironment. The reasons why this fibroblast activation becomes chronic are not understood but eventually lead to fibrosis of the skin and internal organs. Fibrosis occurs when the rate of ECM synthesis exceeds the rate of breakdown resulting in deposition of ECM proteins. The first indication that collagen protein production is altered in SSc was demonstrated in a historic study in which explanted SSc fibroblasts were shown to produce more collagen I protein *in vitro* than normal matched controls (2). Collagen I is the principal component of the normal ECM accounting for 80% of matrix protein (12). Increased expression of several additional ECM proteins has been observed in SSc including collagen I, collagen VI, collagen VII, collagen XVI, fibronectin, tenascin, glycosaminglycans, and proteoglycans (12).

Endothelial cell alterations

Although the hallmark of SSc is pathological remodeling of the connective tissues, the disease is believed to originate with an injury to the endothelial cells that becomes aggravated by a chronic inflammatory reaction (1). The nature of the endothelial cell injury in SSc is not known but results in altered levels of several important vasoregulators. ET-1, a vasoconstrictor, is elevated in early and late stage SSc and stimulates vessel wall ECM production and deposition (13). Although the vasodilator nitric oxide is present in SSc tissue, endothelial cell responsiveness to it is

reduced (14). Altered expression of prostaglandins, von Willebrand factor, and tissue plasminogen activator in SSc contributes to an overall activation of the coagulation cascade and concomitant reduction in fibrin clot breakdown. In SSc skin, fibrin clots are commonly observed in thrombotic microvessels (15). Contributing further to the occlusion of blood flow is aggregation of platelets and lymphocytes to the vessel wall because of increased adhesiveness (16). Ultimately intimal proliferation, vessel wall stiffness, enhanced coagulation, and occluded vessels develop (17). SSc vessels begin to degenerate as a result of endothelial cell apoptosis caused by anti-endothelial cell autoantibodies and interactions with killer T cells that induce Fas-mediated or granzyme/perforin-initiated apoptosis (16-18). Failure to deliver oxygenated blood to the tissues leads to ischemia and further increases the injury. These changes are diagnosed as Raynaud's phenomenon, the earliest clinical manifestation of SSc (19).

Attempts to repair endothelial cell damage by the initiation of angiogenesis processes fail. The majority of damage occurs in the microvessels resulting in large avascular areas of SSc skin (19). Blood mononuclear cells isolated from SSc patients have reduced ability to initiate angiogenesis compared to controls (20). Several antiangiogenic factors are elevated in SSc including endostatin, platelet factor 4, thrombospondin, and IL-4 (21-24). Surprisingly, elevated expression of several pro-angiogenic factors is observed as well including VCAM-1, E-selectin, P-selectin, and monocyte chemoattractant protein-1 (25-27). The most potent angiogenic factor, VEGF, and its receptors are also elevated in SSc skin and endothelial cells suggesting a functional defect in responding to angiogenic stimuli in SSc (28-29). Failure to repair

the vascular damage and resolve the inflammatory response leads to pathological alterations in the skin.

Inflammation and the immune response

Activation of the immune system occurs early in SSc. Currently it is not known if the SSc immune response is the initial pathogenic event or if it is secondary to other processes (30). In affected skin of SSc patients, infiltration of T cells, macrophages, mast cells, and occasionally, B-lymphocytes in patients with recent onset (31). Lymphocytic infiltration precedes the development of fibrotic lesions and the degree of infiltration determines the severity of fibrosis (32). Multiple alterations in the adaptive and humoral immune systems have been detected in SSc patients.

Chemokines recruit mediators of the adaptive immune response to the site of injury. CD4+ helper T cells and macrophages are the most prominent mononuclear cells in SSc skin (33). Oligoclonal expansion of T cells in situ in SSc skin suggests activation by a specific antigen although its identity has not been discovered (34). Peripheral blood T cells in SSc express the interleukin-2 receptor, indicative of T-cell activation (3). Activated T cells produce Th2 cell-derived cytokines IL-4, IL-5, IL-10, IL-13, IL-17, and IL-21 (35-37). IL-4 is a major fibrogenic cytokine of SSc that increases collagen production by fibroblasts and promotes production of TGF-β (30). The presence of a Th1 cytokine signature is detectable in some SSc patients having an increased percentage of IFNγ positive peripheral blood T cells (38). Th1 and Th2 responses contribute to the adaptive immune response in SSc.

The humoral immune response in SSc is manifested in the activation of B cells indicated by hypergamma-globulinaemia, the production of autoantibodies, overexpression of the B cell transduction molecule CD19 in peripheral blood, expanded naïve B cells, and diminished but chronically activated memory B cells (39). The presence of autoantibodies occurs in 90% of patients with SSc. Antibodies against nuclear proteins include anti-Scl-70 directed against topoisomerase I, anticentromere, and anti-polymerase I and III auotantibodies. Additionally, anti-fibribllarin, antiendoethelial cell, and antifibroblast antibodies have all been detected in SSc patient sera (1,30). Although autoantibodies are common in SSc, their role in pathogenesis is not understood. The resolution of both the adaptive and humoral immune responses in SSc skin does not occur and persistant inflammation leads to alterations in endothelial cells and fibroblasts.

Pulmonary Arterial hypertension

Pulmonary Arterial Hypertension (PAH) is a severe and often fatal complication of SSc (10-16%), which occurs more frequently in patients with limited disease (40-41). PAH is characterized by an increase in blood pressure in the pulmonary artery or lung vasculature. Clinically, it is defined as a sustained elevation in pulmonary arterial pressure above 25 mm Hg at rest and above 30 mm Hg during exercise, with a mean pulmonary-capillary wedge pressure and left ventricular end-diastolic pressure below 15 mm Hg. PAH has been classified into three groups: idiopathic (cause unknown), familial (inherited), and secondary (associated with a variety of other conditions, such as infection with the human immunodeficiency virus, portal hypertension, anorexigen use, congenital heart disease, and connective tissue diseases like SSc) (42). Recently progress in diagnosis and treatments have been significantly improved quality of life of PAH patients, but for reasons that are not clearly understood, SSc-PAH patients have worse prognosis compared with other forms of PAH (43 .

PAH has been recognized as a complex, multi-factorial condition involving numerous biochemical pathways and different cell types. It is a severe vascular disorder characterized by vasoconstriction of small pulmonary arteries, thrombosis and inflammation. Progressive intimal and medial thickening, due to proliferation and migration of vascular smooth muscle cells and fibroblasts, reduces the cross-sectional area of the pulmonary microvasculature, causing alterations in pulmonary resistance. The normal pulmonary endothelium maintains a low vascular resistance, suppresses vascular smooth muscle growth, inhibits platelet adherence and aggregation, and stems

inflammation. In patients with PAH, the endothelium has lost these vasoprotective functions (44-46)

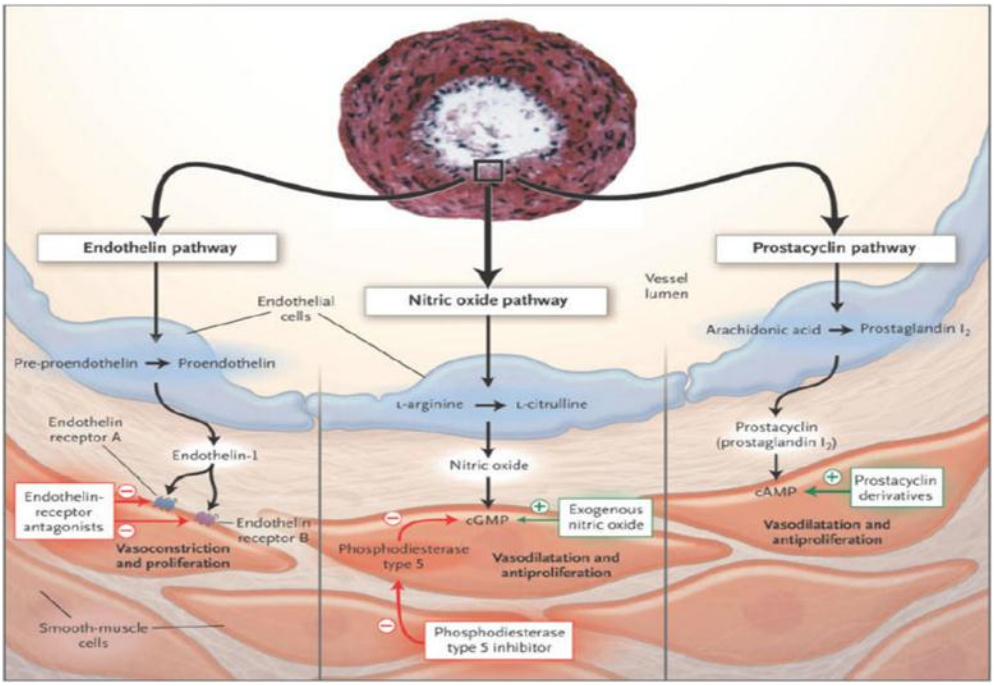


Figure 1.3. Three main pathways involved in Pulmonary Arterial Hypertension.

The chief vascular alterations occurring in PAH are vasoconstriction, SMC and EC proliferation, and thrombosis. These observations indicate the presence of imbalances between vasodilators and vasoconstrictors

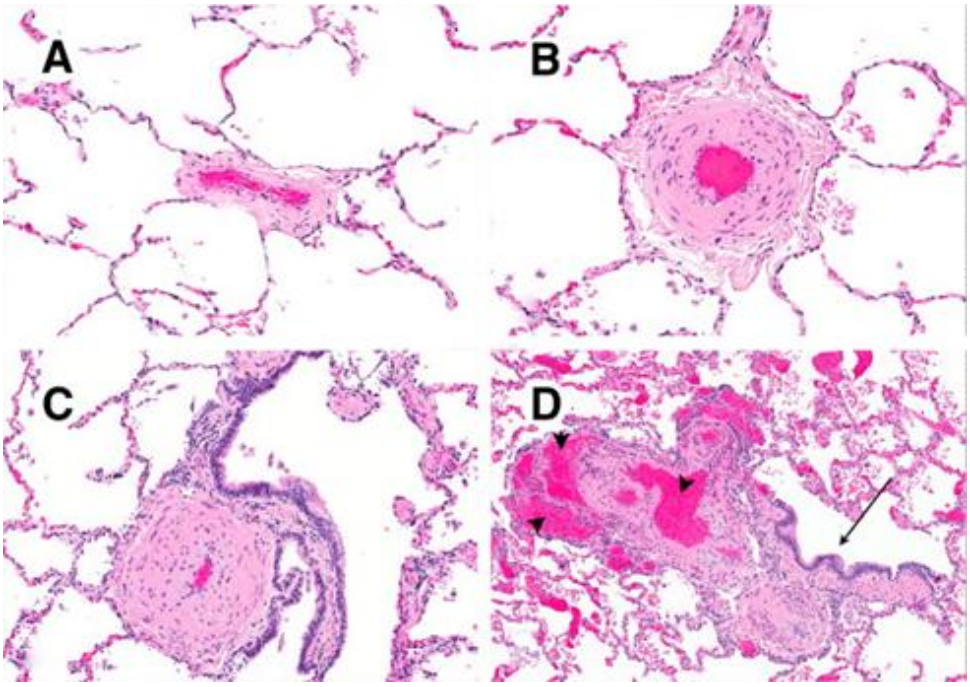


Figure 1.4. **Phases of lesions in pulmonary arterial hypertension (PAH).**

(A) Normal intra-acinar artery. (B) Intra-acinar artery with markedly thickened media, presumably caused by extension of proliferating smooth muscle cells into distal vessels. (C) Concentric intimal thickening, cellular (laminar) type. (D) Plexiform lesion. There is a proliferation of small vessels with surrounding ectatic vessels filled with blood (arrowheads). The lesion is adjacent to a bronchiole (arrow).

Current theory suggests that endothelial dysfunction occurs early in disease pathogenesis, leading to reduced production of vasodilators, such as nitric oxide (NO) and prostacyclin, and increased elaboration of vasoconstrictors, mitogens, and prothrombotic and proinflammatory mediators (such as thromboxane, endothelin, plasminogen activator inhibitor, and 5-lipooxygenase). (47-49). Vascular remodeling itself involves all layers of the vessel wall and is characterized by proliferative and obstructive changes involving many cell types, including endothelial cells, smooth muscle cells and fibroblasts. These structural changes of the pulmonary vascular bed resulting in an increase in pulmonary vascular resistance

In SSc-PAH patients, the presence of inflammatory cell infiltrates in perivascular regions of pulmonary arteries that have been detected more frequently than other forms of PAH (50-51). Immune cells are currently considered to be important mediators of PAH as a source of various cytokines and chemokines that contribute to the pathological vessel remodeling.[8] Furthermore, recent studies described histological differences in pulmonary vessels of SSc-PAH when compared to idiopathic PAH (IPAH) (51-53).

These findings suggest that in SSc patients, combinations of an enhanced inflammatory milieu together with the systemic vascular dysfunction are likely to contribute to the distinct manifestation of PAH.

Endothelin-1

Endothelin-1 (ET-1) is a potent vasoconstricting peptide produced predominantly by endothelial cells, but it is also produced by leukocytes, macrophages, smooth muscle cells, cardiomyocytes and mesangial cells. It is a key regulator of vascular homeostasis. It is overproduced in pulmonary arterial hypertension and is a critical component of the disease progression. (54-58) The excess of ET is associated with dramatic structural changes in the pathology of PAH vasculature, including inflammation, vasoconstriction, cell proliferation, and fibrosis.

ET-1 genes (pre-pro-ET-1 genes) code for a large precursor-protein mRNA (pre-pro-ET-1 mRNA). Different stimuli modulate the transcription of the pre-pro-ET-1 gene. The translation of pre-pro-ET-1 mRNA results in the formation of a 203-amino acid pre-proET-1 peptide, which is cleaved to the 38-amino acid peptide big ET-1. Big ET-1 is transformed to ET-1 through cleavage bond by ET-converting enzyme-1 (ECE-1).

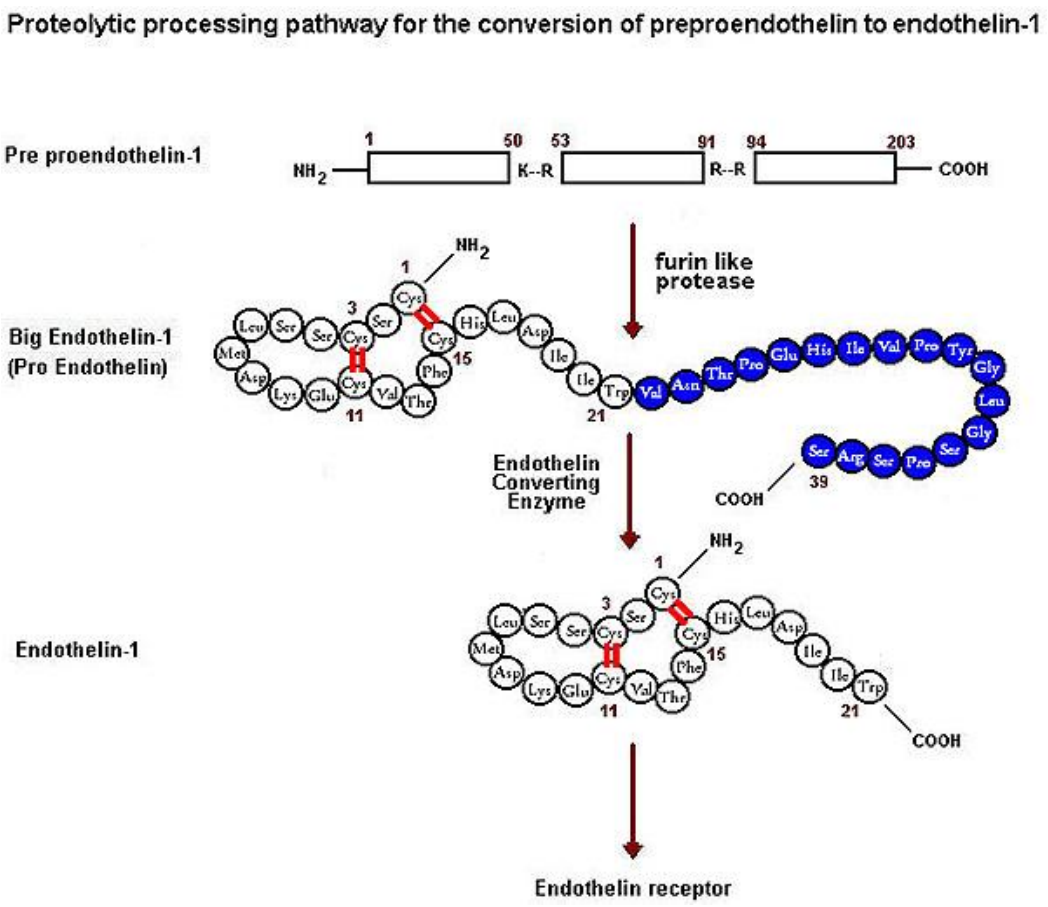


Figure 1.5. ET-1 structure.

ET-1 genes (pre-pro-ET-1 genes) code for a large precursor-protein mRNA (pre-pro-ET-1 mRNA). Different stimuli modulate the transcription of the pre-pro-ET-1 gene. The translation of pre-pro-ET-1 mRNA results in the formation of a 203-amino acid pre-proET-1 peptide, which is cleaved to the 38-amino acid peptide big ET-1. Big ET-1 is transformed to ET-1 through cleavage bond by ET-converting enzyme-1 (ECE-1).

Endothelin-1 exerts its effects by binding to two distinct cell surface ET receptors, ET_A and ET_B. Both receptors belong to the G protein-coupled receptor (GPCR) family and mediate biological responses from a variety of stimuli. ET_A-receptors may play a role in the maintenance of basal vasomotor tone and blood pressure in humans due to an increase in cytosolic calcium level via influx of extracellular calcium and release from intracellular stores (59-60). ET_A-receptors are coupled to Gq/11, G12/13 and Gi heterotrimer G protein subunits which link to phospholipase C, RhoA-GTPase and adenylyl cyclase (AC) inhibition, respectively (61).

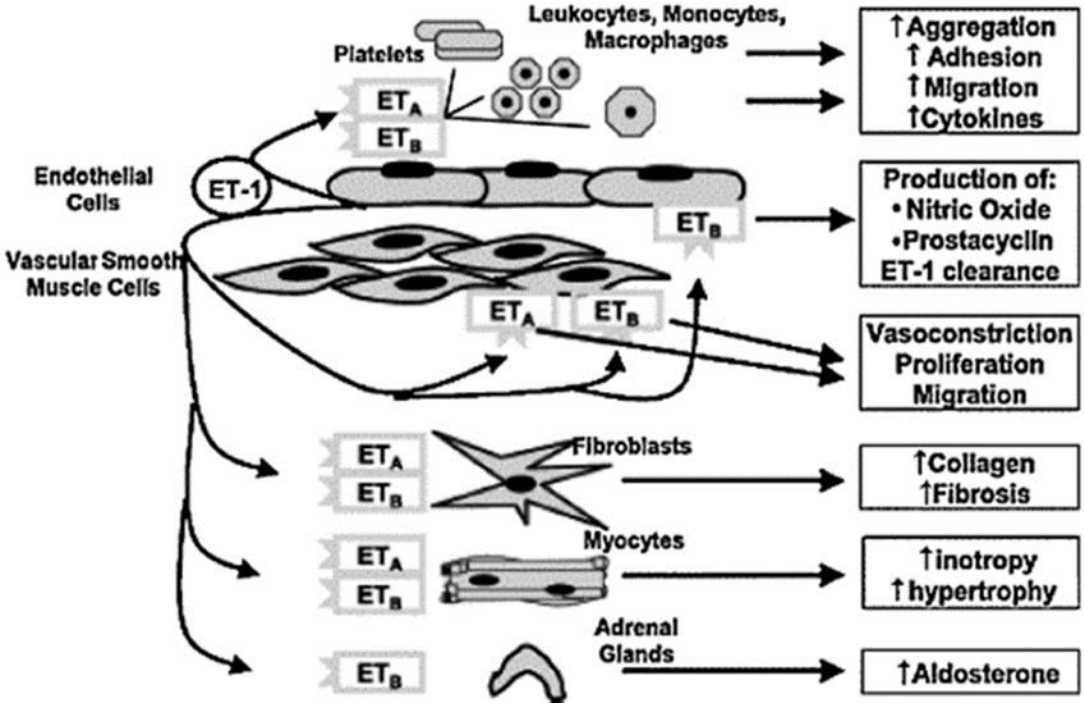


Figure 1.6. Main functions of Endothelin 1

Regulation of ET-1 production

Regulation of ET-1 gene expression has been studied intensely in different experimental models. Several cytokines such as thrombin, TGF-β, tumor necrosis factor-β, interleukin-1 and insulin have been shown to increase ET-1 production in cultured endothelial cells (62-63). These studies implicated several signaling pathways, including [Ca^{2+}] mobilization, PKC, MAP kinase, and cAMP as mediators of ET-1 gene regulation. However, specific mechanisms whereby elevated intracellular $[Ca^{2+}]_i$ levels regulate ET-1 synthesis are still not known. Progress has also been made in characterizing transcription factors regulating ET-1 promoter activity. One of the main regulatory factors is a FOS/JUN complex that binds to an *activator protein 1* (AP-1) response element located at a -108 bp in the ET-1 promoter region. This site mediates upregulation of the ET-1 gene by phorbol esters, Angiotensin II, Thrombin, and *High-density lipoprotein* (HDL), which stimulate AP-1 in a *Protein kinase C* (PKC)-dependent manner. On the other hand, Leptin activates AP-1 through the Jun N-terminal kinase (JNK) and extracellular-signal-regulated kinases 1/2 (Erk1/2) pathways. AP-1 in cooperation with GATA-binding factor 2 (GATA2) is also required for the basal transcription of the ET-1 gene in endothelial cells, while other members of the GATA family regulate ET-1 expression in other cell types. Additional important transcription binding sites include hypoxia response element, Hypoxia-inducible factors (HIF-1), transforming growth factor β (TGF-β)/Smad response element, which have been also described to cooperate with AP-1 to induce ET-1 (64) as well as the Nuclear factor kB (NF-κB) binding site that mediates responses to inflammatory cytokines. Other cell type specific response elements have also been characterized (65)

Human leukocyte antigen (HLA)

HLA is the name of the subset of genes within the human major histocompatibility complex (MHC) that encode cell-surface antigen-presenting proteins. The HLA system was initially discovered because of its role in tissue rejection following transplantation and many of the genes have been shown to have important functions in the biology of the immune system (66-69). HLA consists of over 140 known genes, which are located on the short arm of chromosome 6 (6p21.3). Based on its function, HLA is subdivided into two classes. HLA class I contains the classical HLA-A, B and C spread over a region of 2Mb. These genes are involved in the presentation of peptides predominantly derived from intracellular proteins, to CD8+ cytotoxic T cells. HLAs class II (HLA-DP, DQ and DR) are functionally specialized for presentation of short protein fragments (antigenic peptides) mainly derived from extracellular proteins, to the T cell receptor on CD4+ helper T cells.

<u>HLA class I - structure and function</u>

HLA class I molecules are heterodimers, consisting of a single transmembrane polypeptide chain (the α-chain) and b_2 microglobulin. The α chain has three polymorphic domains, α_1, α_2, α_3. The peptide-binding groove which binds peptides derived from cytosolic proteins is located between α_1 and α_2 domains. The α_3 segment is highly conserved and is homologous to Ig constant domains and is non-covalently bound to β_2 microglobulin (β2m). These two components interact with $alpha_1$ and $alpha_2$ domains to maintain their proper conformation. All nucleated cells in the body express class I HLA molecules. The MHC class I proteins display intracellular antigens on the cell surface for

recognition by T cell receptors on $CD8^+$ T cells. These antigenic peptides are primarily generated by proteolysis of endogenous proteins through proteasome and other enzymes (70). Folding of MHC class I heavy chains and assembly into complexes containing β2m and peptide takes place in the endoplasmic reticulum (ER). Newly synthesized heavy chains are translocated into the ER and properly folded with the aid of molecular chaperones calnexin and calreticulin. After binding of β2m, calnexin is then released from the complex. Peptides, which are generated in the cytoplasm are transported to the ER via transporter associated with antigen processing (TAP). Peptide-loading complex also contains tapasin (TSN), thiol oxidoreductase ERp57 and protein disulfide isomerase (PDI). These additional proteins play a role in loading and optimizing peptide binding. After successful peptide loading, MHC class I molecules are released from the peptide-loading complex and exported through Golgi to cell surface.

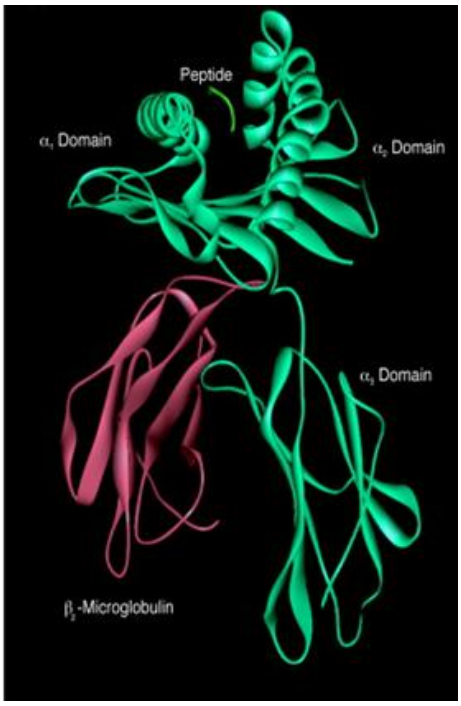


Fig. 1.7. The three-dimensional structure of HLA class I.

Human leukocyte antigen (HLA) class I molecules are heterodimers comprising a heavy chain membrane glycoprotein of ~44 kDa noncovalently bound to a light chain of 12 kDa, termed β_2-microglobulin (β_2m). The heavy chain has three well-differentiated structural regions: a highly hydrophilic intracytoplasmic tail of ~30 amino acids; a hydrophobic transmembrane region of ~25 amino acids; and an extracellular region that contains the groove where the peptides bind. The peptide-binding groove is not a homogeneous space. There are smaller subgrooves known as pockets (six in total, A to F) that accommodate the amino acid chains of the peptide located in the groove. A high degree of homology is found among all HLA class I molecules in the framework regions, but the amino acids adjacent to the peptide binding site are highly polymorphic, thus the size and hydrophobicity of these pockets varies among different HLA class I molecules; they therefore will bind different peptides

The role of HLA polymorphism in disease susceptibility

HLA genes are highly polymorphic with different HLA molecules binding a different set of peptides. The polymorphism within HLA genes maximizes the number of antigenic peptides that can be bound and presented to an individual's T cells. The polymorphism among class I HLA gene products creates variation in the chemical surface of the peptide-binding groove. For any given HLA molecule, binding of a peptide usually requires the peptide to have one or more specific amino acids at a fixed position, frequently the terminal or penultimate amino acid of the peptide. Binding of the specific amino acid in the groove of the HLA molecule occurs in what is termed the anchor site(s). The other amino acids can be variable so that each HLA molecule can bind many different peptides. Other polymorphic residues of the HLA molecule are those in contact with the T cell receptor (TCR), which interacts with both peptide and the HLA molecule itself. Polymorphic loci in the HLA provide useful tools for the study of evolution in man (71-73) and to investigate the contribution of the HLA to genetic disease, particularly of the autoimmune type.

HLA polymorphism influences the ability of different HLA molecules to present endogenous peptides; such differences are believed to underlie most of the associations between HLA class I antigens and susceptibility to diseases (7, 9) or progression of the diseases (74-79). There are two general explanations for HLA and disease associations. First, there may be linkage disequilibrium between alleles at a particular disease-associated locus and the HLA allele associated with that disease. Another possible explanation for these associations is that the HLA antigen itself plays a role in the disease providing a binding site on the surface of the cell for a disease-

provoking virus or bacterium allowing it to enter the cell or resembling the pathogenic molecule so that the immune system fails to recognize it as foreign and fails to mount an immune response against it.

A unique mechanism with a possible relevance to our study was recently proposed to explain pathological contribution of HLA-B27 to ankylosing spondilitis. It appears that HLA-B27 heavy chain exhibits abnormal properties, including a tendency to misfold in the ER triggering ER stress response and activation of unfolded protein response (UPR) (80). ER stress caused by accumulation of unfolded proteins leads to reduced global protein synthesis, induction of chaperons to increase folding capacity of the ER, and translocation of misfolded proteins to the cytoplasm where they undergo proteosome-mediated degradation. The latter process is known as ER-associated degradation (ERAD). On the other hand, prolonged ER stress can lead to cell death (81).

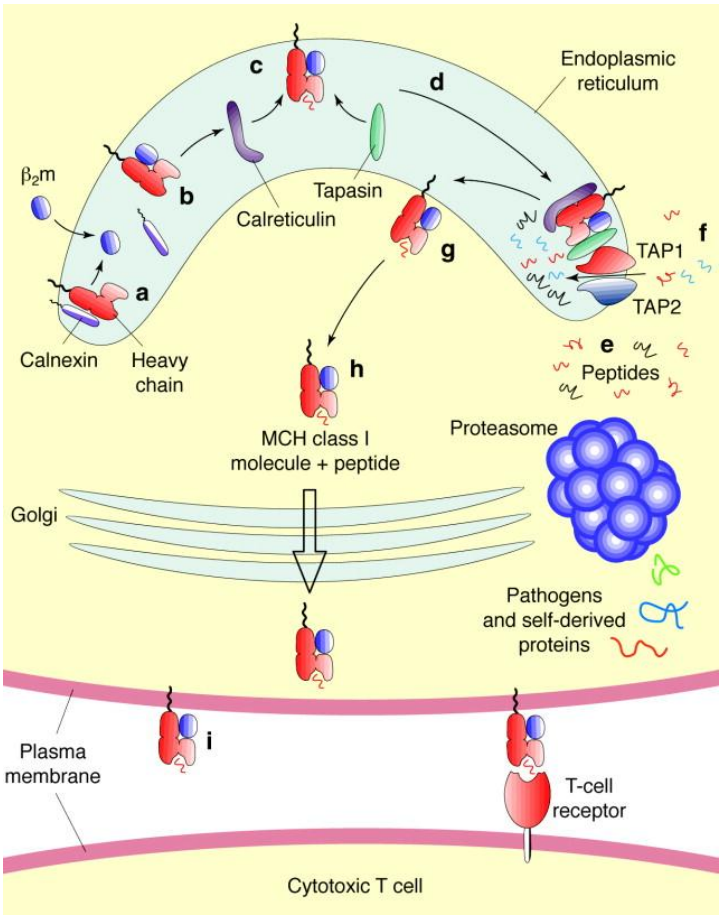


Fig. 1.8. Antigen processing and presentation.

HLA class I molecules is initiated in the endoplasmic reticulum (ER). (**a**) Newly synthesized heavy chain (HC) binds a membrane-bound chaperone known as calnexin, which retains the HLA molecule in a partially folded state in the ER. (**b**) The binding of β_2-microglobulin (β_2m) to the HC dissociates calnexin and the HC–β_2m heterodimer subsequently binds to a complex of proteins, one of which, (**c**) calreticulin, is also a chaperone. (**d**) The transporter associated with antigen processing 1 (TAP1)-associated protein, tapasin, binds to the TAP1 subunit of the TAP complex. The association of tapasin with HC–β_2m allows the heterodimer to await a suitable peptide. (**e**) Cytosolic proteins targeted for destruction are degraded by the proteasome, yielding peptides of 8–12 residues, usually nonamers, which are suitable for HLA class I presentation. (**f**) These peptides are translocated to the ER by the TAP complex. (**g**) When a peptide binds to the HC–β_2m heterodimer, the HLA class I molecule folds properly and is released from the ER, (**h**) entering the secretory pathway to be (**i**) displayed at the cell surface. Usually the peptides displayed at the cell surface are self-proteins and do not initiate an immune response. Only when pathogen-derived proteins appear in the cytosol can they contribute to a pool of HLA–peptide complexes displayed at the surface. These peptides are recognized by specific class I-restricted $CD8^+$ T cells that initiate an immune response against the pathogen.

HLA-B35 association with disease

HLA-B35 is a specific HLA class I antigen. Its frequency depends on ethnic background and varies between 10-20% of total population. In particular 9.9% of European Caucasian, 8.6% of American Caucasian, 12.5% of African American, 22.1% of Oriental American, 7.2% of African, and 9.4% of Japanese carry this allele. HLA-B35 presence is particularly high (15.4%) in the Italian population. Previous studies have found association between HLA-B35 and numerous disorders (82-83), as well as severe viral infections (84-85). The molecular mechanisms underlying these associations are still unknown, but according to some studies the role of HLA-B35 in the rapid progression of infection could be related to the changes in the levels of free cytoplasmatic Ca^{2+} and Mg^{2+} ions (86-87). Studies in patients with HIV infection from different geographical areas have shown a correlation between HLA-B35 phenotype and progression of AIDS (88-97). Among HLA class I alleles, HLA-B*57 and B*27 have consistently been associated with slower rates of disease progression, while HLA-B*35 and B*53 have been associated with more rapid development of AIDS (98-100).

HLA-B35 association with SSc vasculopathy

There is a strong evidence for the contribution of genetic factors to the development of SSc (101) In particular, polymorphism of several loci within the MHC region was consistently found in different cohorts of SSc patients and was recently confirmed in a large scale GWAS study (102). Of particular interest is HLA-B35, which was shown to be associated with increased risk for developing PAH in Italian SSc patients (103-104). Frequency of HLA-B35 depends on ethnic background and varies

between 10-20% of total population. HLA-B35 allele was also found to be strongly associated with SSc in Choctaw Indians (105). Notably, studies of patients with HIV infection have shown a correlation between HLA-B35 allele and a rapid progression of AIDS (106).

ER stress and unfolded protein response

Endoplasmic reticulum (ER) stress and unfolded protein response (UPR) constitute a homeostatic response to accumulation of misfolded protein as well as numerous other stimuli and can occur in various tissues and organs. Although ER stress serves a protective role that allows cells to deal with the noxious stimuli, prolonged ER stress contributes to the development and progression of many diseases, including neurodegenerative disorders, type 2 diabetes, artherosclerosis and cancer (107).

The ER is an intracellular organelle where most of the secretory and membrane proteins are synthesized, post-translationally modified and folded into their correct conformations. Only properly folded proteins can be transported to the Golgi for further processing (108). In addition, the ER is responsible for intracellular calcium homeostasis (109). ER stress occurs when there is an imbalance between protein load and folding capacity, but also can be induced by other mechanisms (110). In response to ER stress, cells activate an adaptive mechanism known as UPR (111). UPR restores protein homeostasis by suppressing protein translation, inducing ER-related molecular chaperones to promote refolding of unfolded proteins, removing unfolded proteins by activating the ER associated protein degradation (ERAD) system, and promoting cell survival. However, during prolonged or overwhelming ER stress when UPR fails to restore the normal function of the ER, a proapoptotic pathway is initiated through the activation of CHOP (CCAAT/enhancer-binding homologous protein) also termed growth-arrest and DNA-damage inducible gene 153 (GADD153) (112).

There are three branches of UPR that are initiated by distinct ER stress transducers located on the ER membrane: (1) PERK, PKR-like endoplasmic reticulum kinase, (2) IRE1, inositol-requiring enzyme 1 and (3) ATF6, activating transcription factor 6. Under basal conditions these proteins are bound by the ER chaperone BiP (immunoglobulin-heavy-chain binding protein, also known as GRP78) and maintained in an inactive state (113). When ER stress develops, BiP is sequestered by the misfolded peptides and, as a consequence, released from the three sensor proteins, which triggers activation of the UPR branches (114). All three branches of the UPR regulate the activation of CHOP, a central mediator of ER stress-induced apoptosis; however, ATF4 is considered to be a major inducer of CHOP expression. CHOP is expressed at a very low level under physiological conditions but its expression level significantly increases in the presence of severe or persistent ER stress. As a transcription factor, CHOP has been shown to regulate numerous pro- and anti-apoptotic genes, including Bcl-2 and GADD34 (115).

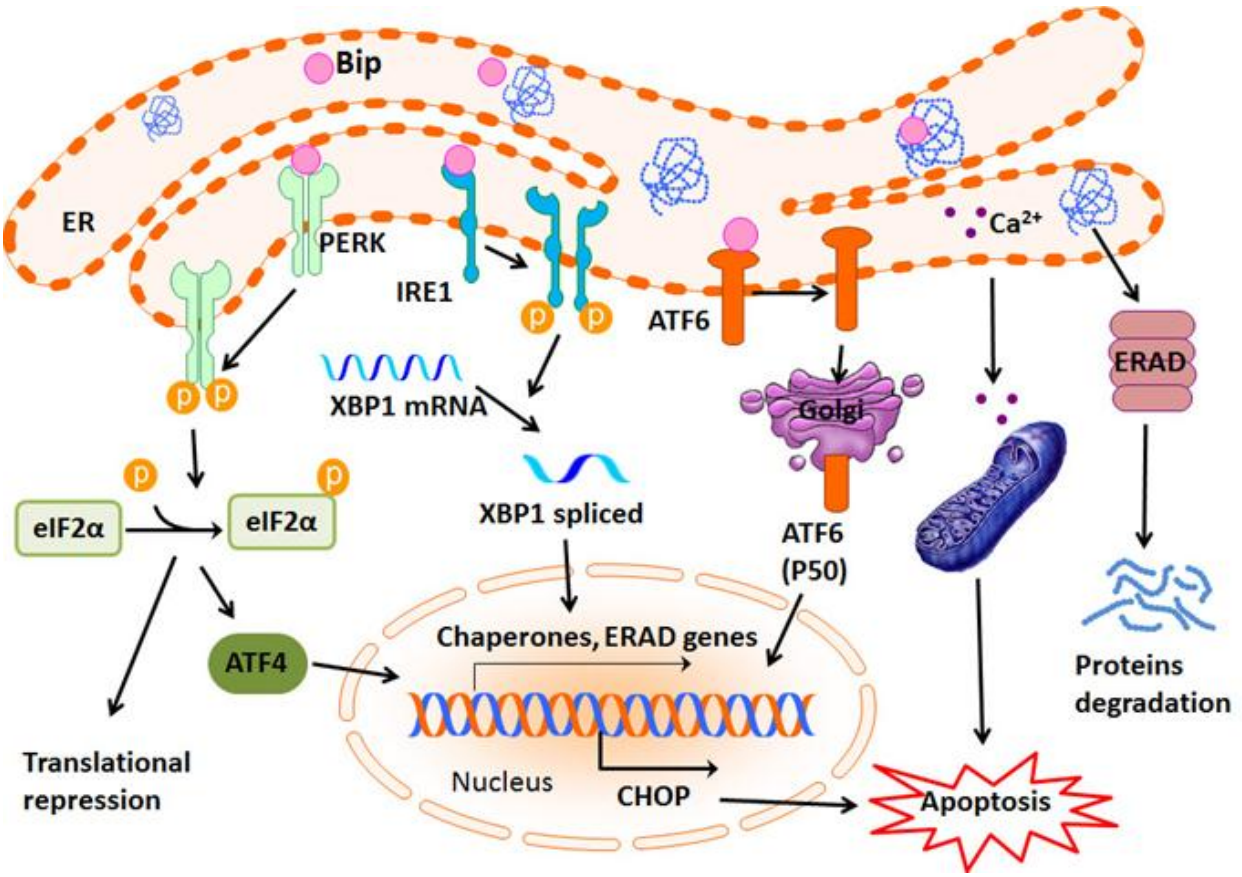


Figure 1.9. Unfolded Protein Responses (UPR) pathways.

Scheme of three UPR pathways: PERK/eIF2α/ATF4 Pathway, IRE1/XBP1 Pathway and ATF6 Pathway.

The PERK/eIF2α/ATF4 Pathway.

When unfolded proteins accumulate in the ER lumen, the first response is to attenuate further protein translation, which reduces the ER load and prevents accumulation of unfolded protein. The PERK/eIF2α pathway mediates this response. PERK, a transmembrane serine/threonine kinase localized in the ER membrane, is activated by ER stress via dimerization and autophosphorylation leading to phosphorylation of eIF2*α* resulting in a global inhibition of translation. Phosphorylated eIF2*α* promotes expression of the selected proteins such as transcription factors ATF4. In response to long-term adaptation to stress conditions, phosphorylation of eIF2*α* induces the expression of the growth arrest and DNA damage gene, GADD34, an important component of translational recovery during the ER stress response (116-119).

The IRE1/XBP1 Pathway.

During ER stress, IRE1 dissociates from BiP, becomes activated and induces splicing of XBP1. The newly generated spliced XBP1 is an active transcription factor, which can induce downstream genes, such as ER chaperones and proteins involved in ER-associated protein degradation (ERAD). These proteins work together to restore the ER homeostasis and promote cell survival (119).

The ATF6 Pathway.

ATF6 is a type II ER transmembrane protein. Like IRE1 and PERK, ATF6 binds to BiP and remains in an inactive state in unstressed cells. In response to ER stress, the BiP/ATF6 complex is dissociated, resulting in the translocation of ATF6 from the ER

membrane to the Golgi where it is cleaved by two serine proteases to produce the active form. The active ATF6 then moves to the nucleus and directly induces transcriptional activation of chaperone molecules such as BIP/GRP78 and ER stress response element- (ERSE-) related genes through binding their promoters. It also induces other URP genes, such as XBP-1 and CHOP(120-121).

ER stress and endothelial dysfunction

Vascular endothelial cells play a major role in maintaining vascular homeostasis. The endothelium not also provides a physical barrier between the vessel wall and lumen, but also secretes several mediators that regulate platelet aggregation, coagulation, fibrinolysis, and vascular tone. Endothelial dysfunction is associated with various diseases, including hypertension, coronary artery disease, chronic heart failure, peripheral artery disease, diabetes, and chronic renal failure. In these vascular diseases, endothelial dysfunction is characterized by an imbalance between vasodilation and vasoconstriction with a shift toward reduced vasodilation, a proinflammatory state, and prothrombic properties.

In atherosclerosis, in vitro and in vivo evidence showed prolonged ER stress often leads to tissue dysfunction and disease. In particular, chronic ER stress has effect on ECs biology (the cause of EC apoptosis in advanced lesions) and mediate proinflammatory effects in early stage disease.

In human aortic endothelial cell (HAEC) culture, IRE1α, ATF6α, and XBP1 were increased as increased of the UPR effector GRP78 expression was observed. In particular, XBP1 was linked to a decrease of VE-cadherin and endothelial cell apoptosis

suggesting that XBP1 splicing could trigger a form of detachment-mediated cell death. In human microvascualr endothelial cell (HMEC) instead was observed an activation of the ER stress transducers, GRP94 and GRP78/BiP and activation of all three UPR branches response and expression of the proapoptotic factor CHOP. In vivo there are evidence for the activation of adaptive UPR in susceptible ECs through ATF6α and lRE1α but not PERK. In such a scenario, additional risk factors may be required to trigger PERK activation.

In patients with diabetes, endothelial dysfunction appears to be a consistent finding and ER stress response results in vascular endothelial-cadherin suppression, apoptotic process is detachment of endothelial cells. Several studies suggest that endothelial cells exposed to elevations and reductions of blood nutrients, high glucose, disrupted Ca^{2+} homeostasis, ROS generation, can go in an apoptosis which may be induced by ER stress response mediators. Both GRP78 and PERK pathway are sensitive to glucose concentration and shown to be induced in endothelial cells. In particular PERK activated an antioxidant program though ATF4 and NRF2 activation. NADPH oxidase, the prime source of ROS in endothelial cells is activated in the presence of high glucose with a concomitant decrease in the generation of NO. NADPH oxidase (Nox1/Nox2) activation might link ER stress and oxidative stress to the high glucose-induced apoptosis of endothelial cells. Nox2 activation and oxidative stress further amplify CHOP/GADD153 induction, which in turn promotes apoptosis. Reports suggest that CHOP induction and apoptosis as a response to ER stress is reduced in Nox2-deficient mice thereby preventing renal dysfunction. This might be true for high glucose-exposed endothelial cells where Nox2 activation has shown to induce

apoptosis which might be possibly through the activation of CHOP-mediated ER stress response. The ER stress response-conferred insulin resistance in endothelial cells could also further promote inflammatory stress signaling and contribute to the metabolic deterioration that is associated with type II diabetes and vascular diseases.

Overall Hypothesis:

ER stress and UPR play a pathogenic role in SSc-PAH by sensitizing endothelial and immune cells. HLA-B35 may further intensify the disease process by contributing to endothelial cell dysfunction, as well as to activation of immune system in patients with PAH.

This work attempts to understand a possible role of HLA-B35/ER stress in the development/progression of PAH disease in SSc patients. Hopefully, characterization of the role of ER stress and its link with disease will enable targeted drug therapy treatment in SSc-PAH patients

Aim 1 : To determine the role of HLA-B35 and ER stress/UPR in endothelial cell function

Specific Aim 1.1: We will determine whether HLA-B35/ER stress affects stimulation of ET-1 expression

Specific Aim 1.2: To determine whether ER stress/UPR markers are elevated in skin biopsies from patients with SSc-PAH

<u>Aim 2 :</u> To determine the role of ER stress/UPR and HLA-B35 in activation of immune cells

Specific Aim 2.1: To characterize ER stress/UPR signature in PBMCs obtained from lcSSc patients with PAH

Specific Aim 2.2: To determine whether signature of ER stress/UPR correlate with the known disease markers in PBMCs

Specific Aim 2.3. To determine whether HLA-B35 contributes to activation of immune cell

Chapter 2

HLA-B35/ER stress induce endothelin-1 via activation of ATF4 in human microvascular endothelial cells

INTRODUCTION

Endothelin-1 (ET-1) is a potent vasoconstrictor and one of the key regulators of vascular homeostasis. ET-1 dysfunction is associated with a number of pathological conditions including hypertension, atherosclerosis, cardiovascular disorders, and cancer (56,57,107). Under physiological conditions, ET-1 is produced in small amounts mainly in endothelial cells (ECs). However in pathophysiological conditions, its production is stimulated in a large number of different cell types, including endothelial cells, vascular smooth muscle cells, cardiac myocytes, and inflammatory cells such as macrophages and leukocytes. In addition to its main role as a vasoconstrictor, ET-1 also contributes to inflammation, as well as fibrosis during various pathophysiological processes.

Extensive studies of ET-1 gene expression have led to characterization of the signaling pathways and transcription factors involved in its regulation (62,63). A complex network consisting of the common and tissue specific transcription factors responding in the coordinated fashion to physiological and pathological stimuli have been shown to regulate ET-1 expression in a cell type and context specific manner. One of the main regulatory factors is a FOS/JUN complex that binds to an *activator protein 1* (AP-1) response element located at a -108 bp in the ET-1 promoter region. This site mediates upregulation of the ET-1 gene by phorbol esters, Angiotensin II, Thrombin, and *High-density lipoprotein* (HDL), which stimulate AP-1 in a *Protein kinase C* (PKC)-dependent manner. On the other hand, Leptin activates AP-1 through the Jun N-terminal kinase (JNK) and extracellular-signal-regulated kinases 1/2 (Erk1/2) pathways. AP-1 in cooperation with GATA-binding factor 2 (GATA2) is also required for the basal transcription of the ET-1 gene in endothelial cells, while other members of the GATA

family regulate ET-1 expression in other cell types. Additional important transcription binding sites include hypoxia response element, Hypoxia-inducible factors (HIF-1), transforming growth factor β (TGF-β)/Smad response element, which have been also described to cooperate with AP-1 to induce ET-1(64), as well as the Nuclear factor □B (NF-κB) binding site that mediates responses to inflammatory cytokines. Other cell type specific response elements have also been characterized (65).

Reticulum (ER) stress is defined as accumulation of unfolded or misfolded proteins in the ER, triggering an adaptive program called the unfolded protein response (UPR). The UPR alleviates ER stress by suppression of protein synthesis, facilitation of protein folding via induction of ER chaperones, and reinforced degradation of unfolded proteins. Three major transmembrane transducers of ER stress have been identified in the ER. Those are the RNA-dependent protein kinase-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring ER-to-nucleus signal kinase 1α (IRE1α). Activation of PERK leads to phosphorylation of the eukaryotic translation initiation factor 2α (eIF2α), causing general inhibition of protein synthesis. In response to ER stress, ATF6 transits to the Golgi where it is cleaved by the proteases *Site-1 protease* (S1P) and *Site-2 protease* (S2P), yielding a free cytoplasmic domain which functions as an active transcription factor. Similarly, activated IRE1α catalyzes removal of a small intron from an X-box-binding protein 1 (XBP1) mRNA. This splicing event produces an active transcription factor XBP1. If the cell fails to deal with the protein-folding defect and restore homeostasis, a pro-apoptotic CCAAT/-enhancer-binding protein homologous protein (CHOP)-mediated pathway is initiated (107).

We have recently shown that ectopic expression of HLA-B35, an antigen associated with SSc in Choctow Indians (105) and SSc-PAH in Italian patients (108, 104), led to a significant increase of ET-1 and a decrease of eNOS in cultured endothelial cells (ECs) (106). In addition to ET-1, we have also observed upregulation of interferon-regulated genes and other inflammatory genes in ECs expressing HLA-B35. Furthermore, expression of HLA-B35, but not a control antigen HLA-B8, potently upregulated several cellular chaperones including BiP, HSP70 and HSP40, suggesting an activation of ER stress/UPR in these cells. However, other UPR genes such as ERO1 (ER oxidoreductin 1), and PDI (protein disulphide isomerase), which are involved in oxidative protein folding, as well as a pro-apoptotic UPR mediator, CHOP were not upregulated, consistent with activation of an adaptive phase of the UPR.

The goal of this study was to investigate which mediators are involved in ET-1 gene regulation in response to HLA-B35/ER stress. Here we report that induction of ER stress activate the eIF2α-ATF4 pathway and promote formation of the ATF4/c-JUN complexes. This protein complex in concert with the NF-κB pathway activates ET-1 gene transcription in endothelial cells.

MATERIALS AND METHODS

Reagents

Thapsigargin (TG) was purchased by Sigma-Aldrich (St. Louis, MO). Tissue culture reagents, EBM kit by Lonza (Walkersville, MD). The protease inhibitor cocktail set III and phosphatase inhibitor cocktail set II were purchased from Calbiochem (San Diego, CA). Enhanced chemiluminescence reagent and bicinchoninic acid protein assay reagent were obtained from Pierce Chemical Co. (Rockford, IL). TRI Reagent was purchased from the Molecular Research Center Inc. (Cincinnati, OH).

For western blot, antibodies were used as followed: goat ATF4 and rabbit ATF6 (Santa Cruz Biotechnology, Santa Clara, CA) at a 1:500 dilution; rabbit pPERK and PERK (Santa Cruz Biotechnology, Santa Clara, CA) at a 1:500 dilution, rabbit p-eIF2α and mouse eIF2α Ab (Santa Cruz Biotechnology, Santa Clara, CA) at 1:500 dilution; rabbit cJun and rabbit NF-κBp65 Ab (Santa Cruz Biotechnology, Santa Clara, CA) at a 1:500 dilution; monoclonal β-actin Ab (Sigma-Aldrich) at 1:5000 dilution and mouse Lamin A/C at 1:1000 dilution.

Cell culture

Human dermal microvascular endothelial cells (HDMECs) were isolated from human foreskins using the protocol of Richard et al [18]. Upon informed consent and in compliance with the Institutional Review Board of Human studies, written approval was obtained from Perinatal Committee (IRB number H-29190) of Boston University Medical

School. Briefly, primary cultures of human foreskins were established after the removal of epidermis. Such cultures consist of a mixture of HDMECs, dermal fibroblasts, and some keratinocytes. Subconfluent cultures were treated with tumor necrosis factor-α for 6 h to selectively induce the expression of E-selectin in HDMECs. HDMECs were then purified using magnetic beads coupled to an anti-E-selectin monoclonal antibody. First passage cultures usually consist of >99% HDMECs. A second immunomagnetic purification step ensures homogenous population of HDMECs suitable for long term culturing. Purity of the HDMEC cultures was evaluated using anti-CD31 and anti-von Willebrand factor antibodies. These cells were cultured on collagen-coated 6-well plate in EBM medium supplemented with 10% FBS, EC growth supplement mix at 37°C under 5% CO2 in air. The culture medium was changed every other day. HDMECs harvested between passage 2 and 6 were used for experiments.

Adenoviral constructs

An adenoviral vector expressing HLA-B35 (or Ad-B8) and control green fluorescent protein (Ad-Go) were generated as described earlier [18]. The dose used to transduce human dermal microvascular endothelial cells was 10 multiplicities of infection of the adenovirus (MOI). ECs grown in a 6-well dish were transduced with Ad (Ad-B35/GFP, -B8/GFP, and -GFP), after 48 h cells were collected for RNA analyses or for Western blot.

Real-time PCR

Total RNA was extracted using the guanidiniumthiocyanate-phenol-chloroform method, concentration and purity was determined by measuring OD at 260 and 280 nm using a spectrophotometer. RNA was reversibly transcribed by aid of the first-strand cDNA Synthesis Kit for RT-PCR (Roche Applied Science, Indianapolis, IN). To avoid amplification from traces of possible DNA contamination in the RNA isolation, PCR primers were designed to span introns. All primers were checked for specificity by Blast search. Real-time RT-PCR was performed using IQ SYBR Green Supermix (Bio-Rad, Hercules, CA) and MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad). The amount of template used in the PCR reactions was cDNA corresponding to 200 ng reverse-transcribed total RNA. DNA polymerase was first activated at 95°C for 3 min, denatured at 95°C for 30 s, and annealed/extended at 61°C for 30 s, for 40 cycles according to the manufacturer's protocol. Expression of the housekeeping gene β-actin served as an internal positive control in each assay performed. After measurement of the relative fluorescence intensity for each sample, the amount of each mRNA transcript was expressed as a threshold cycle value. The primers listed in **table 1.**

Table 1. List of primers used for Real-Time PCR

	Forward	Reverse
PPET-1	5′-gctcgtccctgatggataaa-3′	5′-ccatacggaacaacgtgct3′
ATF4	5’-tggctggctgtggatgg-3’	5’-tcccggagaaggcatcct-3’
ATF6	5’-ttttagcccgggactctttc-3’	5’-tcagcaaagagagcagaatcc-3’
XBP1 unspliced	5’-ccttgtagttgagaaccagg-3’	5’-gggcttggtatatatgtgg-3’
XBP1 spliced	5’-ggtctgctgagtccgcagcagg-3’	5’-gggcttggtatatatgtgg-3’
βACTIN	5′-aatgtcgcggaggacctttgattgc-3′	5′-aggatggcaagggacttcctgtaa-3′

Western blot analysis

Cells were collected and washed with PBS. Cell pellets were suspended in lysis buffer containing 20 mM Tris-HCl, pH 7.5, 15 mM NaCl,1mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, and 1 mM glycerophosphate with freshly added phosphatase inhibitors (5 mM sodium fluoride and 1 mM Na3VO4) and a protease inhibitor mixture (Sigma-Aldrich). Protein concentration was quantified using the BCA Protein Assay kit (Pierce). Equal amounts of total proteins per sample were separated via SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked in milk in TBST overnight at 4°C and probed with primary Ab overnight at 4°C. After TBST washes, membranes were probed with HRP-conjugated secondary Ab against the appropriate species for 1–2 h at room temperature. Protein levels were visualized using ECL reagents (Amersham Biosciences, Piscataway, NJ).

ET-1 bio-assay

The ET-1 bioassay was performed according to the protocol supplied with the kit from Assay Designs (cat no. 900-020A). Standards and samples were incubated in supplied pre-coated 96-well plate, washed, incubated with horse radish peroxidase labeled anti- ET-1 antibody and washed again before adding the provided TMB substrate and measuring the absorbance.

siRNA experiments

HDMECs were trasfected with either siRNA specific to human ATF4 (ON-TARGET plus SMARTpool, Dharmacon RNA Technologies, CO), PKR (Santa Cruz biotechnology, CA) or negative-control siRNA (Qiagen, Chatsworth, CA) at concentration of 20nM using HiPerfect reagent (Qiagen) according to the manufacturer's protocol. After 48 hours, RNA was extracted and Real-time PCR was performed.

Inhibitor experiment

HDMECs were incubated in the presence of the 25nM of JNK SP600125 or NF-κB SN50 inhibitor (Enzo Life Sciences, Farmingdale, NY) for 3 hours before treatment. After 48 hours, RNA and protein were extracted.

Co-Immunoprecipitation

Cell lysates were prepared after appropriate treatment in radioimmune precipitation buffer. For immunoprecipitation of cJUN (or NF-κB p65), antibody was added to 300 μg of precleared cell lysate, and complex formation was carried out at 4 °C overnight. The protein-antibody complexes were recovered using protein G-Sepharose beads for 2 h at 4°C. The immunoprecipitates were washed four times in radioimmune precipitation buffer, eluted by boiling for 5 min in 2× SDS sample buffer, and analyzed by Western blot with anti-ATF4 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Plasmids, Transient Transfections and Luciferase Assay

Luciferase reporters driven by −650-bp and −193-bp fragments (representing the wildtype and mutated AP-1 site) of the human ET-1 promoter described previously 1. Transient transfections of promoters were performed in HDMECs seeded into 6-well plates using Fugene6 (Roche Applied Science) according to the manufacturer's instructions. After overnight incubation, cells were treated and then further incubated for 24 h. The cells were harvested and assayed for luciferase reporter activity using the Promega luciferase assay kit according to the manufacturer's instructions. Promoter/reporter plasmids were cotransfected with pCMV-βGal (Clontech), which was used to adjust for differences in transfection efficiencies between samples. Cells were

harvested and Luciferase activity of the promoter was assayed using Promega Luciferase assay kit. Values given are means ± standard errors of triplicate assays from three individual experiments.

Immunostaining

Skin sections biopsies from healthy individuals and limited cutaneous systemic sclerosis (lcSSc) patients including 8 patients with pulmonary arterial hypertension (lcSSc-PAH) based on echoradiography and right heart catheterization and 11 patients without PAH (lcSSc-noPAH) and 5 healthy controls were provided by the Boston University Core Centers. Patient information is included in **Table 1**. Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. Briefly, 8-micrometer-thick sections were mounted on APES (amino-propyl-triethoxy-silane)-coated slides, deparaffinized with Histo-Clear (National diagnostic, Atlanta GA), and rehydrated through a graded series of ethanol. Endogenous peroxidase was blocked by incubation in 0.3% hydrogen peroxide for 15 minutes and incubated with blocking buffer for 1 hour. The sections were then incubated overnight at 4°C with antibody against ATF4 (Abcam, Cambridge, MA) diluted 1:500 in blocking serum, followed by the incubation with secondary antibody. The concentration of primary antibody was first tested to determine the optimal sensitivity range. The immunoreactivity was visualized with diaminobenzidine (Vector laboratories, Burlingame, CA) and the sections were counterstained with hematoxylin.

Statistical Analysis

Student's t test analysis was performed to determine statistical significance. Values less than or equal to 0.05 were considered statistically significant. All experiments were repeated at least three times using independently isolated endothelial cell cultures.

Table 1. Clinical and hemodynamic data of study subjects. PAP = pulmonary artery pressure. PCWP = pulmonary capillary wedge pressure. PASP=pulmonary artery systolic pressure.

	Sample	Age	Gender	Disease Duration	PAP mmHg	PCWP mmHg	PASP	Treatment	ATF4 staining
Normal	11-6	30	M						++
Normal	11-7	24	M						+
Normal	11-8	47	F						++
Normal	12-1	25	M						-
Normal	12-2	25	F						+
lcSSc PAH	12-8	65	F	16 years 1 month	49	30	44	no medications	-
lcSSc PAH	12-7	58	M	16 years	30	12	65	no medications	++
lcSSc PAH	12-6	69	F	16 years	54	5	71	no medications	++
lcSSc PAH	12-3	75	F	75 years	48	9-10	62	sildenafil	+
lcSSc PAH	12-17	30	F	1 years 8 months	25	2	N/A	cell cept revatio	+
lcSSc PAH	12-20	60	F	3 years	25	2	N/A	prednisone	+++
lcSSc PAH	12-18	59	F	15 years	N/A	N/A	N/A	none	+
lcSSc PAH	12-19	52	F	25 years	N/A	N/A	N/A	viagra	++
lcSSc NoPAH	12-27	59	M				45	none	+
lcSSc NoPAH	12-2	65	F	10 years				myocophenolate	+
lcSSc NoPAH	12-10	40	F	2 years				none	++
lcSSc NoPAH	12-1	58	F	2 years				prednisone, mycophenolate	-
lcSSc NoPAH	12-35	56	F	8 years			33	none	+
lcSSc NoPAH	12-31	61	F	14 years				hydroxychloroquine	+
lcSSc NoPAH	12-28	65	F	6 months			25	none	-
lcSSc NoPAH	12-25	67	M	7 years			25	none	+
lcSSc NoPAH	12-10	40	F	2 years				cyclophosphamide	++
lcSSc NoPAH	12-13	39	F	1 year				cytoxan	+
lcSSc NoPAH	12-21	20	F	6 years				none	++

- indicates no staining or little staining in <10% of the cells

+ indicates faint, partial staining in >20% of the cells

++ indicates light to moderate stain in >50% of the cells

+++ indicates bright staining in >50% of the cells

RESULTS

HLA-B35/ER stress activate common ER stress/UPR pathways in HDMECs

In the initial experiment we compared the effects of HLA-B35 with a known ER stress inducer, thapsigargin (TG) on the expression of ET-1 mRNA and protein in primary dermal microvascular endothelial cells (HDMECs). HLA-B35 was expressed using adenoviral delivery as previously described [16]. To control for the presence of adenoviral genes we used adenovirus expressing a closely related antigen, HLA-B8, as well as an empty virus. As shown in **Fig. 2.1a**, HLA-B35 upregulated (pre-pro-endothelin-1) PPET-1 mRNA levels with a similar potency 7-fold ± 0.58, p = 0.05 vs 8-fold± 0.25, p=0.05, respectively, while TG was a stronger inducer of PPET-1 (26-fold ± 0.75, p = 0.001). Ad-HLA-B8, as well as an empty virus vector (data not shown) did not affect PPET1 mRNA expression. To verify that the increase in PPET-1 mRNA corresponds to an increase of the bioactive 21-aa ET-1 peptide, we measured levels of ET-1 protein in supernatants of Ad-B35 (and Ad-B8) and TG treated ECs **(fig.2.1b).** Consistent with the mRNA measurements ER stress inducers increased ET-1 protein levels (Ad-B35, 3.5-fold± 0.6, p=0.05 and TG, 5.3-fold± 0.80, p=0.05).

To further characterize the nature of the HLA-B35-mediated ER stress, we examined the effect of HLA-B35 (or HLA-B8) and TG on the mRNA expression of the three main UPR mediators, transcription factors ATF4, ATF6 and XBP1. ATF4 mRNA levels were significantly increased in response to the HLA-B35, while TG was a less potent inducer of ATF4 mRNA under this experimental conditions (**Fig. 2.2a**). Furthermore, both HLA-B35 and TG moderately increased spliced (active) form of the

transcription factor XBP1 (XBP1s) (**Fig. 2,2c)**. In contrast, the expression level of ATF6 was not responsive to any of these treatments in HDMECs (**Fig.2.2b**).

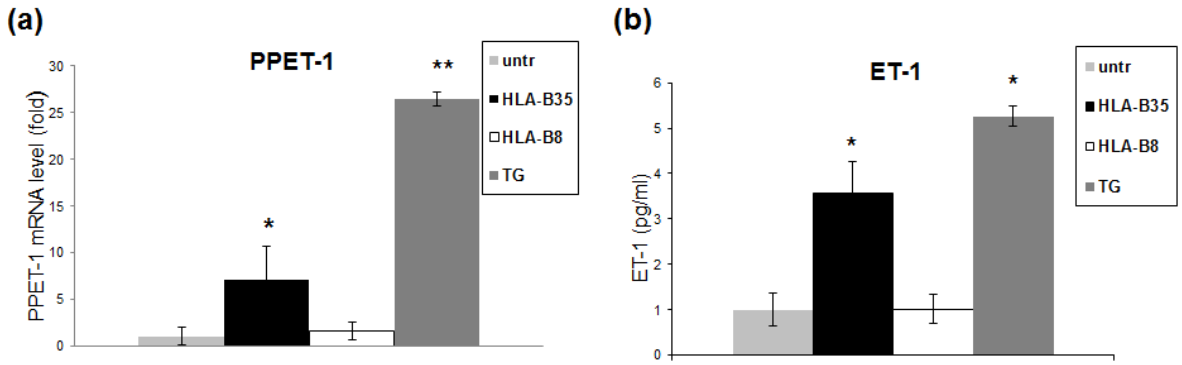


Figure 2.1. HLA-B35 and TG upregulate ET-1 mRNA and protein in HDMECs

Upregulation of PPET-1 mRNA after HLA-B35 (or HLA-B8) and TG treatments alone (a) in HDMECs. Confluent dishes of HDMECs were transduced with 10 MOI of Adenovirus encoding HLA-B35/GFP (Ad-HLA-B8/GFP) for 48 h or treated with 10pM TG for 24 hours. Total RNA was extracted and mRNA levels of PPET-1 were quantified by quantitative RT-PCR. Expression of the housekeeping gene β-actin served as an internal positive control in each assay performed. After measurement of the relative fluorescence intensity for each sample, the amount of each mRNA transcript was expressed as a threshold cycle value. (b) Bioactive 21-aa ET-1 peptide in HDMECs after Ad-B35/GFP (Ad-B8/GFP), TG. ET-1 protein was measured by ELISA in the supernatants. The average protein concentration for each group is represented as a bar ± SE. *p = 0.05; **p = 0.001

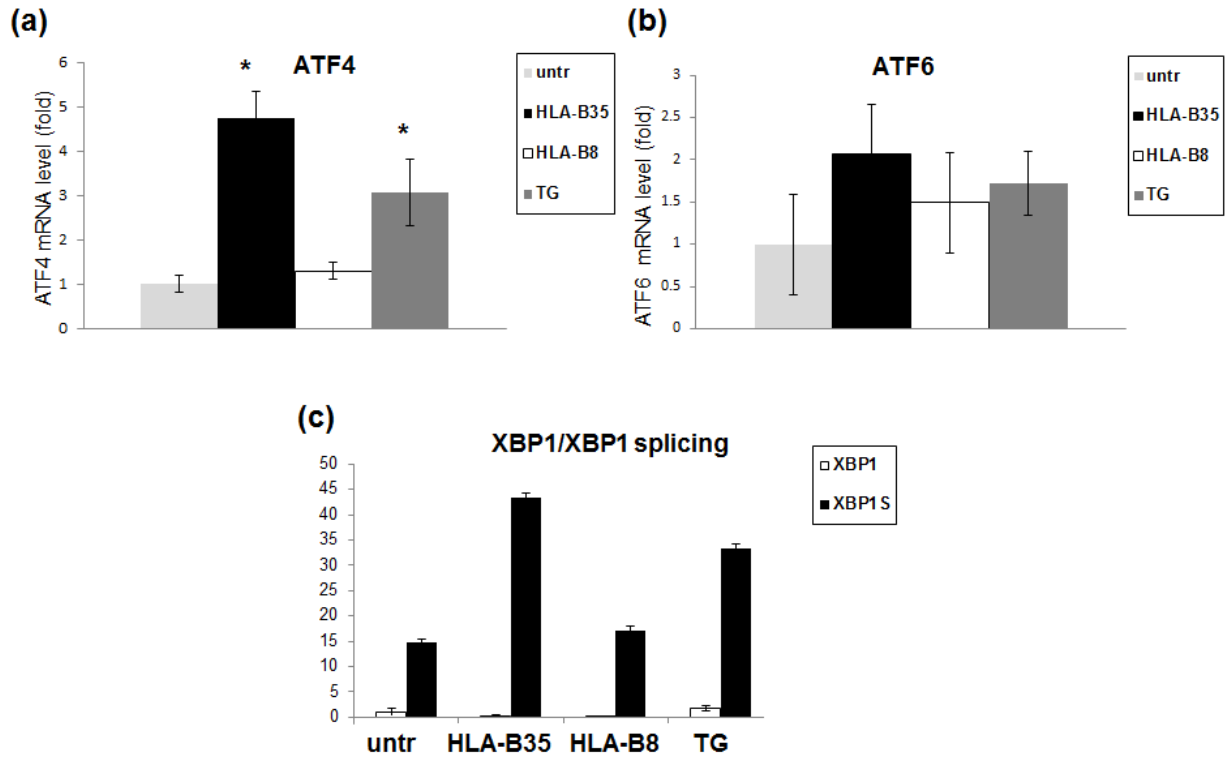


Figure 2.2. HLA-B35/ER stress activate selected ER stress/UPR pathways

ATF4 (a), ATF6 (b) and XBP1 splicing (c) levels in HDMECs treated with HLA B35 (HLA-B8) and TG. Confluent dishes of HDMECs were transduced with 10 MOI of Ad-B35/GFP (or Ad- B8/GFP) for 48 h treated with 10pM TG for 24 hours. Total RNA was extracted and mRNA levels of transcription factors were examined by quantitative RT-PCR. Expression of the housekeeping gene β-actin served as an internal positive control in each assay performed. After measurement of the relative fluorescence intensity for each sample, the amount of each mRNA transcript was expressed as a threshold cycle value. *p = 0.05; **p = 0.001

Since nuclear translocation of ATF4 is indicative of its activation status, we examined nuclear extracts for the presence of ATF4 by western blot. Nuclear ATF4 was examined at various time points (15 min. to 6 hours) after TG treatments and 24 hours post infection with HLA-B35 or HLA-B8 adenoviruses. Nuclear ATF4 was rapidly increased (15-30 min) after TG **(Fig. 2.3a).** Likewise, HLA-B35 markedly increased nuclear presence of ATF4 **(Fig. 2.3b).** Furthermore, increased phosphorylation of the upstream activators of ATF4, PERK and eIF2α, was observed in response to these treatments (Fig. 3, right panels). Consistent with the mRNA data, nuclear levels of ATF6 remained unchanged. Together, these results demonstrate that ATF4 is activated in a similar manner by HLA-B35 and ER stress/UPR inducer in endothelial cells.

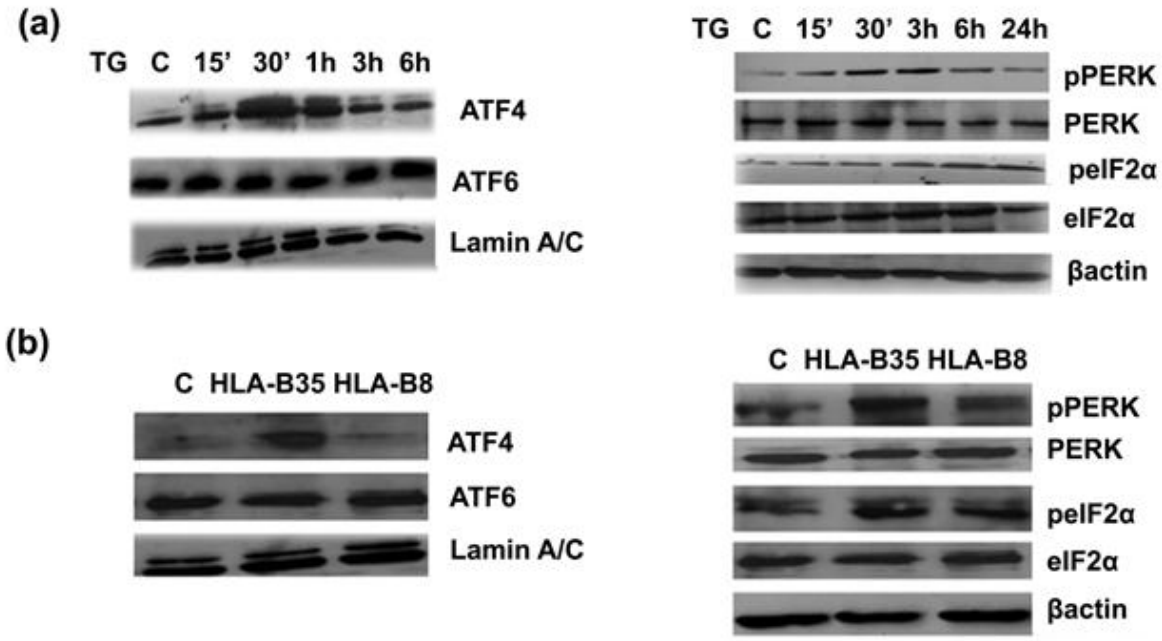


Figure 2.3. HLA-B35/ER stress activate ATF4 nuclear translocation and enhance phosphorylation of PERK and eIF2α

ATF4**,** pPERK/PERK, and p-eIF2α**/**eIF2α protein levels in HDMECs after treatment with 10pM TG (a) or transduction with 10 MOI of HLA-B35 or HLA-B8 Ads for 48 hours (b). 20 μg of nuclear extract were separated via 15% SDS-PAGE for ATF4 and 10% SDS-PAGE for ATF6. 20 μg of total cellular proteins were separated via 15% SDS-PAGE for pPERK/PERK and 10% SDS-PAGE for peIF2α/eIF2α, then transferred to a nitrocellulose membrane. The blots were probed overnight with primary Abs at 4°C. As a control for equal protein loading, membranes were stripped and reprobed for Lamin A/C or β-actin. Representative blots from at least three independent experiments are shown.

ATF4 is required for the upregulation of ET-1 in response to HLA-B35/ER stress a in HDMECs

In order to determine whether ATF4 is directly involved in the regulation of ET-1 gene we employed a siRNA approach to knock down ATF4. Initial experiments established an optimal dose and time for the ATF4 siRNA to achieve maximal inhibition of endogenous ATF4 mRNA level. Treatment of HDMECs with 20nM ATF4 siRNA for 48 hours resulted in depletion of ATF4 mRNA levels (up to 50-60%) **(Fig 2.4a).** Under these conditions basal expression levels of ET-1 mRNA and protein were also consistently decreased by ~30% **(Fig. 2.4b and c).** Following 24 hour incubation with siRNA, cells were treated with Ad-B35/GFP (or Ad-B8) and TG for additional 24 hours. Depletion of ATF4 completely abolished upregulation of ET-1 mRNA **(Fig. 2.4b)** and protein **(Fig. 2.4c)** in response to these treatments, suggesting that ATF4 is required for these responses.

We next examined the distribution of ATF4 protein in the human skin by immunohistochemistry. Analyses of skin microvessels showed heteregoneus distribution of ATF4, with some vessels exhibiting strong endothelial cell nuclear staining, while other vessels were negative for ATF4 **(Fig. 2.4d).** The number of positive vessels also varied between different individuals. Because patients with SSc have elevated circulating levels of ET-1 [20] we also analyzed skin samples obtained from 19 patients with limited cutaneous SSc (lcSSc), including 8 patients with PAH (lcSSc-PAH), and 11 lcSSc-noPAH. Similar to healthy control skin, endothelial cell expression of

ATF4 varied between the patients, however there was no overall difference in the intensity or staining pattern between lcSSc and healthy individual biopsies. We did not have information regarding the level of circulating ET-1 or the presence of HLA-B35 antigen in this group of patients. Together, these data indicate that ATF4 is highly expressed in a subset of dermal microvessels, where it is likely involved in responses to various environmental stimuli.

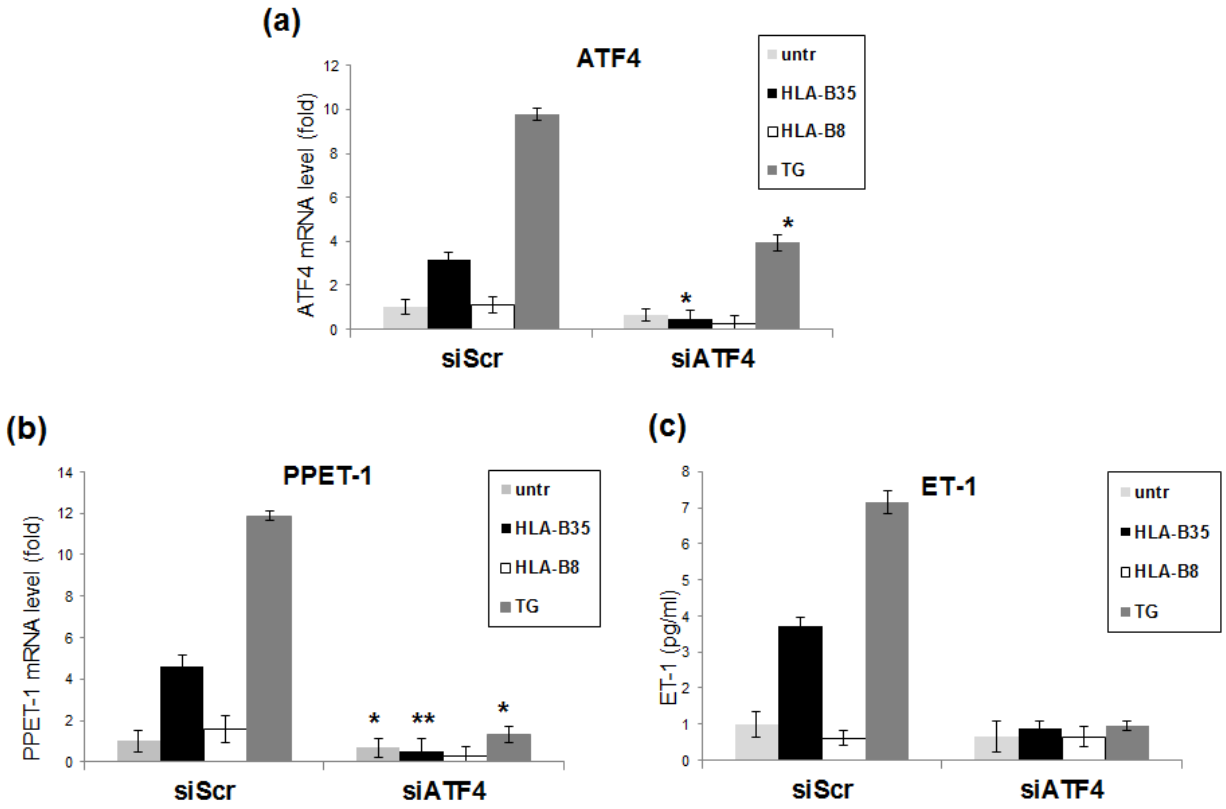


Figure. 2.4. HLA-B35/ER stress upregulate ET1 via ATF4

80% confluency, HDMECs were treated with 20nM ATF4 siRNA (or siSCR) prior to treatment with HLA-B35 (HLA-B8) and TG. Total RNA was extracted and mRNA level of ATF4 (a) and PPET-1 (b) were quantified by quantitative RT-PCR. Expression of the housekeeping gene β-actin served as an internal positive control in each assay performed. After measurement of the relative fluorescence intensity for each sample, the amount of each mRNA transcript was expressed as a threshold cycle value. (c) ET-1 protein was measured by ELISA in the supernatants (n=2). The average ET-1 protein concentration for each group is represented as a bar ± SE. *p = 0.05; **p = 0.001.

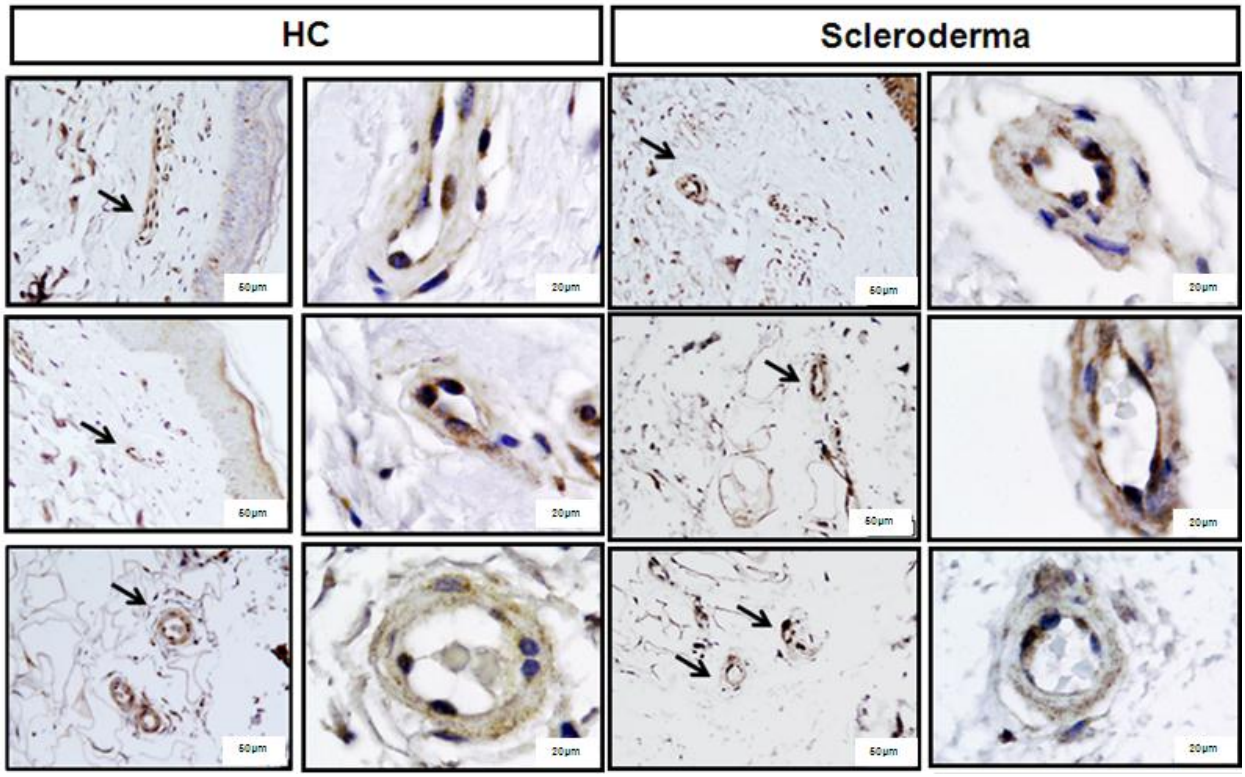


Fig. 2.4 (d). ATF4 protein expression in human skin microvessels.

Lesional skin biopsies were obtained from patients with lcSSc (with and without PAH) and healthy controls, and processed for immunohistochemistry as described under Methods. Representative images of microvessels from healthy control and lcSSc patients is shown; similar immunostaining pattern was observed in control and lcSSc skin biopsies. Bar: 50µm, 10 µm

JNK and NF-κB contribute to HLA-B35/ER stress induction of ET-1

JNK and NF-κB pathways have been previously reported to contribute to the ET-1 gene expression in response to various stimuli [4,5]. To determine if JNK and NF-κB contribute to the upregulation of ET-1 in response to ER stress, cells were treated with HLA-B35 and TG in the presence or absence of the pharmacological inhibitors of these pathways. Treatment with JNK inhibitor (SP6001, 25 mM) resulted in down regulation of the basal and agonist-induced PPET-1 mRNA levels (Fig. 5a, top panel). On the other hand**,** basal expression of PPET-1 mRNA was not affected by the NF-κB inhibitor (SN50, 25 mM), however stimulation of PPET1 by HLA-B35 and TG was completely inhibited **(Fig. 2.5b, top panel).** Similar results were observed at the protein levels **(Fig. 2.5a and b, bottom panels).** Interestingly, while stimulation of ET-1 by HLA-B35 or TG was similarly affected by the inhibitors of the JNK and NF-κB pathways.

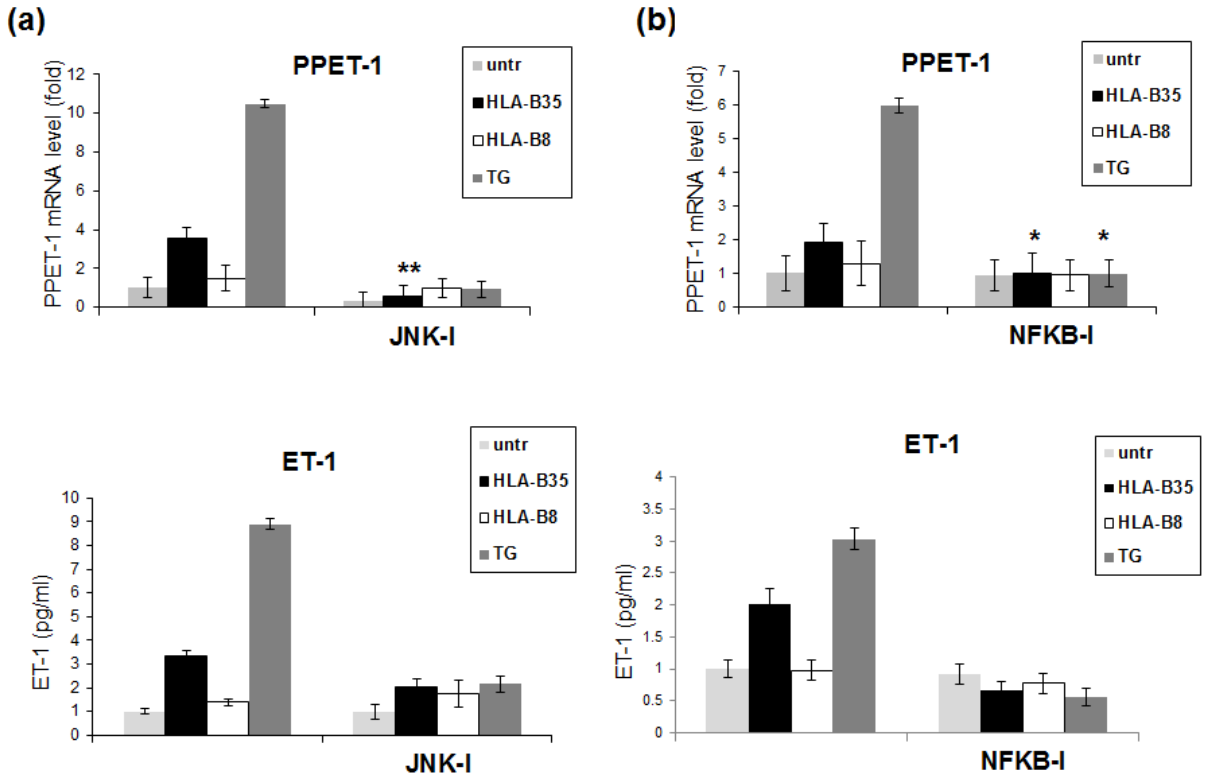


Fig. 2.5. JNK and NF-κB contribute to the HLA-B35/ ER stress induction of ET-1

Cells were treated with 25nM of JNK (a) or NF-κB (b) inhibitors for 3 hours before HLA-B35 (HLA-B8) and TG treatment. Total RNA was extracted and mRNA levels of PPET-1 were quantified by quantitative RT-PCR (top panel). ET-1 protein was measured by ELISA in the supernatants (bottom panel). The average protein concentration for each group (n=2) is represented as a bar ± SE *p = 0.05; **p = 0.001

ATF4/c-JUN complexes mediate ET-1 induction by HLA-B35/ER stress

ET-1 gene promoter contains binding sites for a number of transcription factors, however using bioinformatics tools we were unable to locate consensus ATF4 binding site within the promoter region. Based on the previous report identifying ATF4 as a partner of c-JUN in a two-hybrid screen [21], we asked whether ATF4 could form protein complexes with c-JUN in HDMECs. As shown in **Fig. 2.6a**, ATF4/c-JUN complexes were present in unstimulated cells and were increased upon stimulation with HLA-B35 and TG. While, we could also detect formation of the ATF/NF-κB complexes in unstimulated cells, formation of these complexes was not affected by the agonists **(Fig. 2.6b),** suggesting that formation of the ATF4/c-JUN complexes is not simply driven by the elevated levels of ATF4 in stimulated cells.

We next utilized human ET-1 promoter constructs consisting of the −650/+172–bp fragment fused to the luciferase reporter gene to confirm functional role of ATF4 in regulation of the ET-1 gene. Transcriptional activation of the ET-1 promoter was observed after treatment with HLA-B35 and TG **(Fig. 2.6c).** To analyze whether AP1 binding site was required for the regulation of ET-1 transcription, cells were transfected with the 193-bp ET-1-prom-luc construct (wild type) or the same construct carrying mutated AP-1 binding site. As shown in **Fig. 2.6d**, mutation in the AP1 binding site reduced the ER stress induction of the ET-1 promoter. These results support the functional role of the AP1 complex in the ER stress-mediated induction of the ET-1 gene expression.

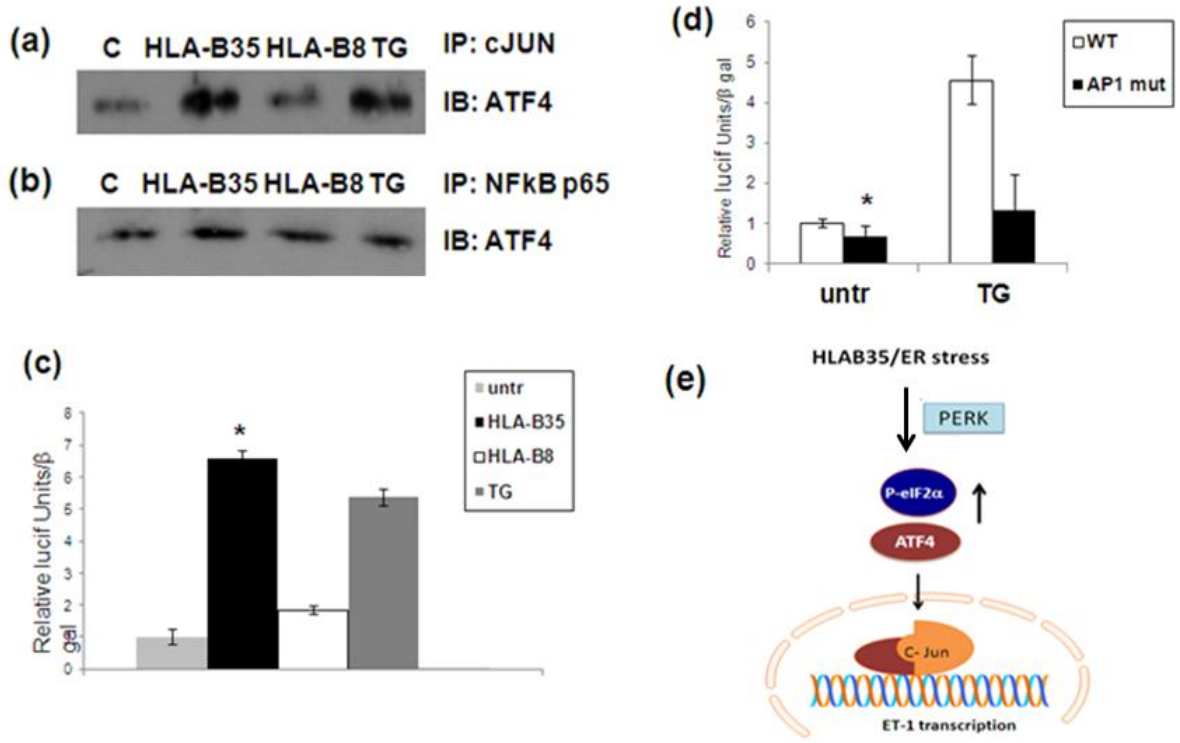


Fig. 2.6. Transcriptional upregulation of ET-1 by HLA-B35/ER stress is mediated through the ATF4/cJUN complex

Cell lysates from the HLA-B35 (HLA-B8) and TG treated HDMECs were immunoprecipitated with cJUN (a) or NF-κB p65 antibodies (b) and then analyzed for ATF4 by western blot. Cells were transfected with the luciferase reporter driven by the −650/+172–bp fragment of the human ET-1 promoter (c) or with the −193-bp ppET-1-prom-luc construct (wild type) or constructs with specific mutations in the AP-1 binding site (d). 24 hours post transfection with the indicated plasmids, cells were stimulated with HLA-B35 (HLA-B8) Ads and TG for an additional 24 h. Transfections were normalized using pSVgalactosidase control vector. Basal and induced luciferase activity was measured by luminometry. The graph represents fold change in promoter activity in response to various treatments in comparison with control promoter, which was arbitrarily set at 1. (e) Schematic diagram showing PERK induced activation of the eIF2α-ATF4 axis followed by the protein complex formation with c-JUN and induction of the ET-1 gene transcription through the AP1 response element.

DISCUSSION

In this study we show for the first time that ATF4 is a novel regulator of the ET-1 gene in endothelial cells. ATF4 contributes to the basal expression of ET-1 and is required for the induction of ET-1 in response to ER stress. Our results strongly suggest that activation of the eIF2α/ATF4 pathway leads to increased formation of the ATF4 protein complexes with c-JUN, which, in turn, activate ET-1 transcription through the AP1 response element. ER stress inducers, including HLA-B35 and TG, also upregulate mRNA and protein expression of ATF4, thus further amplifying this signaling pathway (see diagram, **Fig. 6e**). Additional experiments show that NF-κB, which is also activated by the ER stress in HDMECs, contributes to the activation of ET-1 gene expression. Interestingly, although, ATF4 forms protein complexes with NF-κB in HDMECs, formation of these complexes was not increased by the stimuli used in our study. Since NF-κB plays a key role in activation of the ET-1 gene by cytokines, it is possible that the ATF4/NF-κB complexes are involved in those responses. Together, this study identifies ATF4 as a key mediator of ET-1 gene activation in response to cellular stress.

ATF4 is a short-live, basic region-leucine zipper (bZip) protein that belongs to a family of the ATF/CREB transcription factors (109). Under normal physiological conditions translation of the ATF4 protein is inefficient due to the presence of a short open reading frame in its 5' untranslated region; however ATF4 protein translation is facilitated by various stress conditions that trigger global inhibition of protein synthesis (109). Such conditions, including ER stress, viral infection, nutrient starvation, and low

levels of heme induce activation of distinct protein kinases that in turn lead to phosphorylation of a common downstream mediator, eIF2α□□resulting in translational repression. In a related study Gargalovic et al have reported activation of the eIF2α-ATF4 in human atherosclerotic lesions and in cultured aortic endothelial cells exposed to oxidized phospholipids (110). The authors demonstrated that ATF4 contributed to the upregulation of several inflammatory cytokines in cultured aortic endothelial cells. ATF4 was also upregulated by herpesvirus 8 infection and contributed to proangiogenic response via MCP1 upregulation (111). Furthermore, rapid induction of ATF4 has been observed in smooth muscle cells (SMCs) in the medial compartment of balloon injured rat carotid arteries [24]. Additional studies with cultured SMCs have demonstrated that *Fibroblast growth factors* (FGF)-2 and mechanical injury stimulate ATF4 levels, and that ATF4 is required for the FGF-2 mediated upregulation of *Vascular endothelial growth factor* (VEGF)-A (112). Our study indicates that ET-1 is among the target genes positively regulated by the eIF2α-ATF4 axis in response to ER stress in endothelial cells. Collectively, these studies support a key role for the eIF2α-ATF4 pathway in response to vascular injury (113).

Endothelial cells constitute a first line of defense protecting tissues from injury. Elevated production of ET-1 is a common characteristic associated with endothelial cell dysfunction in various pathological conditions, including pulmonary arterial hypertension (114). Previous studies have shown that HLA-B35 is associated with an increased risk for developing PAH in patients with scleroderma (SSc) (104). The current study further supports the potential pathogenic role of HLA-B35 in upregulating ET-1 production and

clarifies the molecular mechanism involved in this process in endothelial cells. In addition, this study raises an intriguing possibility that chronic activation of the eIF2α/ATF4 pathway could contribute to the disease pathogenesis. Although, we were able to demonstrate, activation of ATF4 in selected skin biopsies of patients with lcSSc, absence of full clinical data, including levels of circulating ET-1 and presence of HLA-B35 antigen, precluded proper analyses of these samples. This limitation may be addressed in future studies with a larger set of fully characterized samples from patients with lcSSc.

Chapter 3

Increased expression of ER stress genes in patients with limited cutaneous Systemic Sclerosis and Pulmonary Arterial Hypertension

INTRODUCTION

Pulmonary Arterial Hypertension (PAH) is a complex, multi-factorial condition involving numerous biochemical pathways and cell types resulting in alterations in vascular reactivity, vascular structure, and interactions of the vessel wall with circulating blood elements (114). PAH is a serious and often fatal complication of SSc, occurring primarily in patients with the limited cutaneous form of the disease (lcSSc), and is one of the main causes of morbidity and mortality in these patients (115-117). It has been suggested that autoimmunity/inflammation and systemic vasculopathy could contribute to the development of lcSSc-PAH, but the underlying mechanisms have not been fully elucidated (43).

Notably, inflammatory cell infiltrates have been observed in perivascular regions of pulmonary arteries more frequently in SSc-PAH than in other forms of PAH (41, 118). The cytokines and chemokines released by these immune cells could contribute to the pathological vessel remodeling in PAH (43).

The Endoplasmic Reticulum (ER) is a multifunctional organelle, which coordinates protein folding, lipid biosynthesis, calcium storage and release. Perturbations that disrupt ER homeostasis lead to ER stress and activation of signaling cascades termed the Unfolded Protein Response (UPR). The main UPR branches include PERK (protein kinase RNA-like ER kinase); IRE1 (inositol requiring protein–1) and ATF6 (activating transcription factor–6). Under basal conditions these specialized ER membrane-associated sensor proteins are bound by the ER chaperone BiP (immunoglobulin-heavy-chain binding protein, also known as GRP78) and maintained in

an inactive state. Accumulation of misfolded proteins in the ER lumen activates adaptive UPR mechanisms through the release of BiP from the sensor proteins and initiation of specific cellular responses aimed at the restoration of ER homeostasis (119). When activated, PERK oligomerizes and phosphorylates itself and the ubiquitous eukaryotic translation initiation factor 2 alpha (eIF2α), which results in global attenuation of influx of proteins to the ER, while selected mRNAs, including ATF4 (activating transcription factor–4), are preferentially translated. PERK also phosphorylates nuclear erythroid 2 p45- related factor (NRF2) involved in restoration of redox balance (120). The transmembrane kinaseendoribonuclease IRE1, governs splicing of the mRNA encoding X-box binding protein–1 (XBP1), a potent transcription factor involved in regulation of genes controlling ER-associated degradation (ERAD), synthesis of ER chaperones, as well as the phospholipid synthesis required for ER expansion. Because of its regulatory role in protein folding and degradation XBP1 is active during the early cytoprotective phase of ER stress, but its activity declines during prolonged ER stress (121). The third arm of the UPR is regulated by ATF6, which upon release from BiP translocates to the Golgi where it is cleaved by S1P and S2P proteases. A released N-terminal ATF6 fragment moves to the nucleus and in concert with XBP1 activates ER stress target genes, including BiP (119-121). Chronic or severe ER stress activates UPR-dependent apoptotic death. While not yet fully understood, this process is associated with PERK-eIF2α mediated transcription activation of C/EBP-homologous protein (CHOP; also known as GADD153), which then downregulates expression of the antiapoptotic mediator B cell lymphoma 2 (BCL-2) and upregulates expression of proapoptotic BIM. Importantly, recent studies point to a more complex regulation of the intensity and

kinetics of UPR signaling depending on the nature of the cellular stress and input from other cellular pathways (121). Accumulating evidence indicates that ER stress is associated with a range of diseases, including neurological disorders, diabetes, metabolic disease, intestinal inflammation and autoimmunity making ER stress a probable instigator of pathological cell death and dysfunction (122-126). Interestingly, there is also evidence that ER homeostasis is closely related to regulation of inflammatory gene expression. A link between ER stress and the inflammatory response was reported in different experimental models, including endothelial cells and immune cells (124, 109, 127, 106). Inflammation can be triggered by chronic excess of metabolic factors, cytokines, and hormones, and those factors can also trigger ER stress, which

can further disrupt metabolic function, leading to more inflammation (106,128).

We have recently reported that ectopic expression of HLA-B35, an antigen associated with SSc in Choctow Indians (104) and SSc-PAH in Italian patients (107, 103), led to changes in the expression of genes related to ER stress in cultured endothelial cells (ECs) (105). Furthermore, activation of the ER stress/UPR pathway correlated with upregulation of interferon-regulated genes and other inflammatory genes in ECs expressing HLA-B35. The goal of this study was to further explore the potential contribution of ER stress/UPR to the pathogenesis of lcSSc-PAH by examining expression levels of the ER stress/UPR genes in PBMCs obtained from patients with lcSSc. Herein, we showed for the first time that expression of selected ER stress/UPR genes is significantly elevated in PBMCs from lSSc patients, with the highest levels observed in patients with PAH. Importantly, there was a positive correlation between ER

stress markers and severity of PAH (PAP) and between ER stress markers and IL-6 levels in lcSSc PBMCs, suggesting that activation of ER stress/UPR, might drive inflammation in PAH associated with SSc.

MATERIALS and METHODS

Study Participants.

Subjects included normal healthy controls (HC, n=36) and patients with lcSSc(n=66), according to criteria established by LeRoy et al. (2). Patients with lcSSc were stratified into those with or without PAH based on echocardiography or right heart catheterization (RHC); all patients designated as PAH were confirmed by RHC. Patients were considered not to have PAH (n=34) if echocardiogram demonstrated a pulmonary artery systolic pressure <35mm Hg and normal right ventricular size and function. Subjects with mean pulmonary arterial pressure ≥ 25mm Hg, a pulmonary capillary wedge pressure (PCWP) ≤15 and a pulmonary vascular resistance ≥3 Wood units by RHC were considered to have PAH (n=32). LSSc patients with mildly elevated pulmonary capillary wedge pressure (PCWP) (>15 to ≤18) were included in our primary analyses consistent with the REVEAL registry with similar rationale (129). Patients that had a mild increase in PCWP included in the primary analysis all had significantly elevated pulmonary vascular resistance (PVR), and significant increases in both the pulmonary artery diastolic minus pulmonary capillary wedge pressure (PAd-PCWP) gradient (>10) and the transpulmonary gradient (>15). Thus, each was considered to have PAH by the pulmonary hypertension expert caring for the patient. Patient characteristics are listed in **Table 1**.

Table 1.Clinical and hemodynamic data of study subjects.

PAP = pulmonary artery pressure. PCWP = pulmonary capillary wedge pressure. CO/CI = Cardiac output (L/min)/ cardiac index (L/min/m2). PVR= pulmonary vascular resistance. ILD = interstitial lung disease. ILD was defined as present (Y=yes) or absent (N=no) based on high resolution computed tomography assessment of the lungs.FVC (%) = estimated forced vital capacity. DLCO = carbon monoxide diffusing capacity.SPAP = estimated systolic pulmonary artery pressure by echocardiogram.

	Sample	Age	Sex	PAP	PCWP	CO/CI	PVR	ILD	FVC	DLCO	HAP	HGB	HGB on	DLCO adj	PASP	Disease	ANA	pattern
		(yrs)		(mmHg)	(mmHg)				(%)	(%)	PAH y/n	g/dL	PFT y/n	for Hgb	(mmHg)	duration	titer	
lcSSc-noPAH	s-y-19	36	F					N	97	61	N	NA		NA	20	20 yrs	1::640	centromere
lcSSc-noPAH	s-y-102	43	F					N	107	83	N	NA		NA	NORMAL	4 yrs 6 mo	1::1600	speckled
lcSSc-noPAH	s-y-116	57	F	21				N	100	66	N	13	N	66.84	35	29 yrs	1::320	centromere
lcSSc-noPAH	s-y-35	46	F					N	65	82	N	NA		NA	NORMAL	25 yrs		
lcSSc-noPAH	s-y-52	61	F					Y	82	69	N	NA		NA	33	10 yrs 2 mo	1::2560	centromere
lcSSc-noPAH	s-y-55	60	F					N	96	61	N	NA		NA	22	18 yrs 4 mo	1::640	centromere
lcSSc-noPAH	s-y-58	44	F	18	10	5/3.7	114	N	110	95	N	14.3	Y	92.54	20	18 yrs	1::5120	anticentromere
lcSSc-noPAH	s-y-67	45	F					N	108	94	N	NA		NA	30	10 yrs	>1::2560	centromere
lcSSc-noPAH	s-y-75	55	F					N	90	98	N	NA		NA	30	10 yrs	1::1280	centromere
lcSSc-noPAH	s-y-80	51	F					N	105	82	N	11.2	Y	88.63	NORMAL	5 yrs	1::1280	centromere
lcSSc-noPAH	s-y-81	37	F					N	na	na	N	NA		NA	25	10 yrs		
lcSSc-noPAH	s-y-82	76	F	21	13	5.1/3.0	125	N	96	51	N	11.2	N	55.12	35	7 months	1::1280	centromere
lcSSc-noPAH	s-y-86	51	F					Y	42	61	N	12.1	N	63.7	NORMAL	7 yrs		
lcSSc-noPAH	s-y-87	38	M					N	95	90	N	15.2	N	88.54	32	1 yr	1::1280	nucleolar
lcSSc-noPAH	s-y-91	56	F					Y	117	65	N	NA		NA	30	5 yrs		
lcSSc-noPAH	s-y-98	56	F					N	89	82	N	NA		NA	25	11 yrs		
lcSSc-noPAH	s-y-182	65	F					Y	64	35	N	NA		NA	NORMAL	15 yrs		
lcSSc-noPAH	s-y-41	61	F	13	1			Y	88	49	Y	14.8	N	47.09		6 yrs	1::120	nucleolar
lcSSc-noPAH	s-y-138	54	F					N	97	69	N	12.6	Y	70.8		1 yr 3 mo	1::1280	homogenous
lcSSc-noPAH	s-y-12	33	F					N	119	94	N	14.4	N	91.31		4 yrs 3 mo	1::2560	centromere
lcSSc-noPAH	s-y-47	69	F					N	96	76	N	13.4	N	76		10 yrs		centromere
lcSSc-noPAH	s-y-0 149	56	F					N	88	52	N	12.3	N	53.91	29	17 yrs		
lcSSc-noPAH	s-y-0 201	71	F	19	12	4.73/2.44	118		84	76	N	NA		NA	40	3 yrs	1::160	antinuclear body
lcSSc-noPAH	s-y-0 215	61	F						82	73	N	NA		NA	NORMAL	3 yrs		
lcSSc-noPAH	s-y-0 221	57	F	21	8	5.69/3.69	183	N	65	52	N	12.1	Y	54.3	39	15 yrs	1::160	speckled
lcSSc-noPAH	s-y-0 227	61	F					Y	72	72	N	11.6	N	76.6	22	5 yrs	1::1280	homogenous
lcSSc-noPAH	s-y-0 228	54	F					N	89	62	N	NA		NA	33	13 yrs	1::2560	centromere
lcSSc-noPAH	s-y-0 254	57	F					Y	79	77	N	12.4	N	79.56	27	2 yrs	1::320	homogenous
lcSSc-noPAH	s-y-1 148	50	F						94	71	N	14.6	N	68.6	NORMAL	8 yrs	1::640	nucleolar
lcSSc-noPAH	s-y-1 160	57	F					Y	67	43	N	NA		NA	NORMAL	9 yrs		
lcSSc-noPAH	s-y-1 172	50	F					Y	78	68	N	NA		NA	NORMAL	4 yrs 2 mo	1::640	centromere
lcSSc-noPAH	s-y-2 100	41	F						87	105	NA	NA		NA	NORMAL	15 yrs		
lcSSc-noPAH	s-y-3 127	41	F					N	101	107	N	NA		NA	26	11 yrs	1::320 & 1::80	antinuclear
lcSSc-noPAH	s-y-3 52	65	F					Y	92	51	Y	NA		NA	34-39	7 yrs	1::2560	centromere

Table 1.Clinical and hemodynamic data of study subjects.

PAP = pulmonary artery pressure. PCWP = pulmonary capillary wedge pressure. CO/CI = Cardiac output (L/min)/ cardiac index (L/min/m2). PVR= pulmonary vascular resistance. ILD = interstitial lung disease. ILD was defined as present (Y=yes) or absent (N=no) based on high resolution computed tomography assessment of the lungs.FVC (%) = estimated forced vital capacity. DLCO = carbon monoxide diffusing capacity.SPAP = estimated systolic pulmonary artery pressure by echocardiogram.

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		(yrs)		(mmHg)	(mmHg)				(%)	(%)	PAH y/n	g/dL	PFT y/n	for Hgb	(mmHg)	duration	titer	
lcSSC-PAH	s-y-173	63	f	44	15	7.0/3.7	331	N	90	49	Y	13.4	N	49	57	1 yr	1::1280	centromere
lcSSC-PAH	s-y-66	80	F	52	16	3.4/2.0	846	N	NA	49	Y	NA		NA		10 yrs		
lcSSC-PAH	s-y-195	66	F	40		3.3/1.9		N	68	23	Y	12.1	N	24.02	50	2 yrs	1::1280	centromere
lcSSC-PAH	s-y-191	67	F	34	15/14	4.5/2.85	356	Y	90	56	Y	12.6	N	57.46	44	4 yrs	1::320	nucleolar
lcSSC-PAH	s-y-130	57	F	25	14			N	100	76	Y					27 yrs	Y	
lcSSC-PAH	s-y-123	68	F	54	18-19	5.0/2.9	543	N			Y					10 yrs		
lcSSC-PAH	s-y-68	55	F	48	13	6.0/3.4	466	N	NORMAL		Y	13.9	N			1 yr		
lcSSC-PAH	s-y-71	63	M	32	10			Y	66	48	Y	12.4	N	51.32		6 yrs	1::80	homogenous
lcSSC-PAH	s-y-101	67	F	26	14						Y					10 yrs	389 units	anti-centromere
lcSSC-PAH	s-y-99	67	F	27	15			N	66	65	Y					20 yrs		
lcSSC-PAH	s-y-94	52	F	25	16			N	107	62	Y	12.5	N	63.84		4 yrs	1::1280	nucleolar
lcSSC-PAH	s-y-0 281	52	F	34	5	6.1/3.28	236	N	102	41	Y	14.3		39.94		1 yr		
lcSSC-PAH	s-y-0 282	62	F	61	3	3.29/2.24	899	Y	86	32	Y				68	11 yrs		
lcSSC-PAH	s-y-0 209	64	F	85		4.04/2.17	931	Y	80	32	Y	12.1	Y	33.42	45-50	30 yrs		
lcSSC-PAH	s-y-1 157	61	M	38	11	7.0/3.41	172	Y	93	67	borderline				37	17 yrs	1::160	speckled
lcSSC-PAH	s-y-3 116	60	F	34	13	5.77/3.35	153	N	100	66	borderline	13	N	66.84	30	28 yrs	1::320	centromere
lcSSC-PAH	s-y-4 24	60	F	72	4	2.7/1.53	1067	Y	97	32	Y	15/6	Y	31.71		7 yrs		
lcSSC-PAH	s-y-29	62	M	58	14	7.4/4.8	475		80	40	Y					4 yrs 2 mo	1::2560	centromere
lcSSC-PAH	s-y-45	52	F	52	11	5.5/3.4	625			48	Y				84	6 yrs		
lcSSC-PAH	s-y-48	63	M	45	10	6.2/3.2	451	Y	75	33	Y	16.3	N	31.58		14 yrs	1::1280	nucleolar
lcSSC-PAH	s-y-54	65	F	34	8	5.5/3.0	378				Y				58	3 yrs	1::640	
lcSSC-PAH	s-y-60	70	F	48	9	4.3/2.4	706		94	50	Y					14 yrs 8 mo	1::640	centromere
lcSSC-PAH	s-y-62	70	F	43	8	3.3/1.8	823		86	44	Y	8.8	N	53.47	55	no data	1::1280	nucleolar
lcSSC-PAH	s-y-64	64	F	33	13	5.4/2.9	222	Y	59	54	Y	13.4	Y	54		3 yrs		
lcSSC-PAH	s-y-83	64	F	37	11	4.1/2.3	519		101	34	Y	10.2	N	38.39		3 yrs	1::640	centromere
lcSSC-PAH	s-y-85	69	M	53	9		720		61	43	Y	13.6	N	42.74	102	1 yr.	1::640	speckled
lcSSC-PAH	s-y-90	56	F	42	5	6.2/3.9	503	Y	99	38	Y				80	10 yrs	1::2560	centromere
lcSSC-PAH	s-y-2	67	F	31	14	5.1/3.3	282		79	58	Y				38	12 yrs 6 mo	1::2560	centromere
lcSSC-PAH	s-y-31	70	F	32	16	5.8/3.2	207		100	68	Y	11.8	N	71.8		10 yrs	1::1280	centromere
lcSSC-PAH	s-y-42	59	F	57	18	4.4/2.5	745	Y			Y					12 years	1::2560	nucleolar
lcSSC-PAH	s-y-89	67	F	37	16	8.2/2.6	205	Y	48	43	Y							
lcSSC-PAH	s-y-0 207	71	F	85	4	4.02/2.28	876		95	44	Y	11.9	N	46.28	65	2 yrs		

Peripheral Blood Mononuclear Cell (PBMC) isolation.

Blood was collected from healthy controls and patients in CPTTM tubes designed for one-step cell separation (Becton Dickinson, Mountain View, CA). The sample was then immediately mixed and centrifuged at 1,800 RCF at ambient temperature for 30 minutes. The PBMC cell layer was then transferred to a 15ml tube, and PBMCs were washed twice with PBS and lysed in RNeasy RLT buffer (Qiagen, Valencia, CA). Healthy control PBMCs were plated in 6 well plates at 8x105-1x106 cells/well in RPMI supplemented with 10% FCS and 1% AA and stimulated with 5 or 10 uM Thapsigargin (TM) for 2, 4, 8, 12 and 24 hours. Per manufacturer's protocol, total RNA was extracted using Qiagen's RNeasy Mini kits, and sent for microarray analysis.

Microarray hybridization and data analysis.

RNA quality was assessed using the Agilent 2100 Bioanalyzer and RNA 6000 Pico Kit. RNA concentration was measured on a Thermo Scientific NanoDrop 2000 Spectrophotometer. Total RNA (25ng) from each sample was converted to Cy3-CTP (Perkin Elmer) labeled cRNA, and Universal Human Reference (UHR) RNA (Stratagene) was converted to Cy5-CTP (Perkin Elmer) labeled cRNA using the Low Input QuickAmp Labeling Kit (Agilent Technologies) for hybridization as described previously (130). Labeled cRNA from UHR and sample were co-hybridized to Agilent Whole Human Genome (4x44K) Oligo Microarrays (G4112F), representing 41,000 unique genes and transcripts. Microarrays were scanned using Agilent's Dual Laser High-Resolution C Scanner. These microarray image files were processed using Agilent's Feature Extraction software, which automatically finds and places microarray

grids, rejects outlier pixels, accurately determines feature intensities and ratios, flags outlier pixels, and calculates statistical confidences. Data were Log2 LOWESS normalized for the Cy5/Cy3 ratio, and data was filtered to select array spots with an intensity 2 fold or greater than the local background in either the Cy3 or Cy5 channel. All data were multiplied by negative one to convert the log10 (C5/C3) ratios to log2 (Cy3/Cy5) ratios. Probes missing more than 20% of the data across all arrays were omitted from further analysis. The time course was time zero transformed by subtracting the mean of the zero time points from all other time points. Gene expression data were organized by average linkage hierarchical clustering using Cluster 3.0 and filtered using 2 arrays, 2-fold cutoff. That resulted in 3,746 probes. This subset was visualized using Java TreeView and partitioned into 1,582 probes upregulated and 2,164 probes downregulated. Agilent probe IDs were converted to Ensembl gene IDs via g:Convert, a part of g:Profiler (131). 1,582 upregulated probes were converted to 1,488 unique gene IDs (94.1% conversion rate) and 2,164 downregulated probes were converted to 1,891 unique gene IDs (87.4% conversion rate). Sets of Ensembl gene IDs were analyzed for functional enrichment via g: GOSt, another component of g:Profiler, in the following annotation categories: Gene Ontology (GO) (132) – biological process (BP), molecular function (MF) and cellular component (CC) and pathways; KEGG (133) and REACTOME (134). Specific functional terms (with the number of annotated genes in the genome below 1,000) were selected in order to account for the biologically informative categories

Quantitative Real-time PCR.

Real-time RT-PCR was performed using IQTM SYBR Green Supermix (Bio-Rad) and MyiQ™ Single-Color Real-Time PCR Detection System (Bio-Rad). The amount of template used in the PCR reactions was cDNA corresponding to 200 ng reverse-transcribed total RNA. DNA polymerase was first activated at 95°C for 3 min., denatured at 95°C for 30 sec., and annealed/extended at 61°C for 30 sec., for 40 cycles according to the manufacturer's protocol. Expression of the housekeeping gene β-actin served as an internal positive control in each assay performed. After measurement of the relative fluorescence intensity for each sample, the amount of each mRNA transcript was expressed as a threshold cycle (ct) value. The primers are available upon request.

Statistical analysis. Comparisons of RT-PCR expression were analyzed by Student's *t*-test. Correlations were tested by Pearson's correlation coefficient

RESULTS

ER stress and UPR genes are upregulated in PBMCs from lcSSc patients

Subjects selected for this study included patients with lcSSc who were recently characterized in a study examining expression of PAH biomarkers (135), as well as an additional lcSSc patients with PAH and lcSSc patients without PAH (**Table 1**). PBMC samples from a total of 34 lSSc patients without PAH (lcSSc-NoPAH), 32 lSSc patients with PAH (lcSSc-PAH) and 36 healthy controls (HC) were analyzed for the presence of ER stress/UPR-related genes. The candidate ER stress/UPR genes consisted of molecular chaperone BiP/Grp78, indicator of the onset of the UPR, as well as key regulators of the UPR pathway including transcription factors ATF4, ATF6, and XPB1. Compared with healthy control subjects, PBMCs from lcSSc -NoPAH patients demonstrated significantly higher expression of BiP ($p<0.0001$). The highest level of BiP was present in samples from patients with lcSSc-PAH when compared to lcSSc-NoPAH samples ($p<0.05$) or HCs ($p<0.0001$) **(Fig. 3.1).** The transcription factors ATF4 and ATF6 were significantly elevated in patients with lcSSc- PAH compared to HCs ($p<0.001$) and lcSSc-NoPAH ($p<0.05$) for ATF6. XBP1 splicing was increased in lcSSc compared with HCs ($p<0.05$), but no significant difference was observed between lcSSc- NoPAH and lcSSc-PAH. Together, these results demonstrate that activation of selected ER stress/UPR in PBMCs is associated with lcSSc and is progressively increased in patients with PAH.

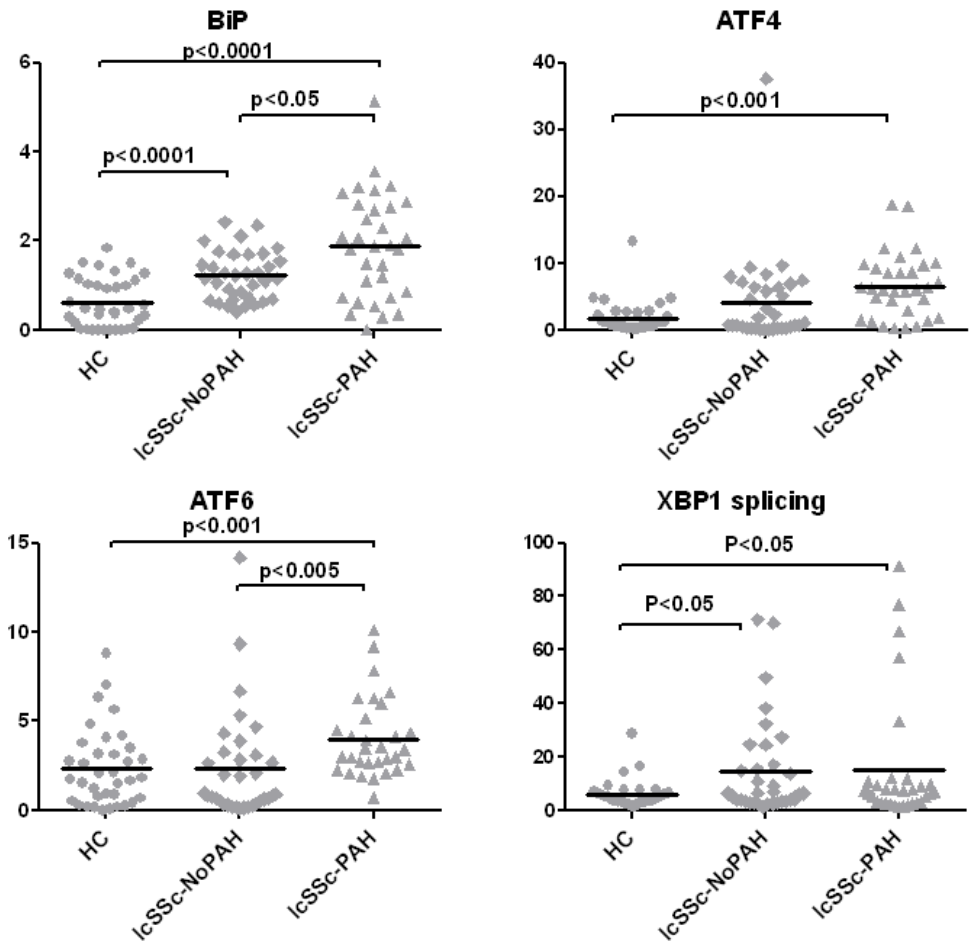

Figure 3.1. Elevated mRNA expression of selected ER stress/UPR markers in patients with lcSSc. PBMCs were isolated from HC (n=36), lcSSc-NoPAH (n=34) and lcSSc-PAH (n=32). mRNA levels of BiP and ATF4 (top panel), ATF6 and XBP1 splicing (bottom panel) were measured by qPCR. Expression of the housekeeping gene β-actin served as an internal positive control. Data are expressed as the fold-change normalized to mRNA expression in HC samples. Each data point represents a single subject; horizontal lines show the mean.

Thapsigargin upregulates heat shock proteins and interferon-regulated genes in PBMCs

Microarray analyses were used to further investigate genes regulated by ER stress in PBMCs. PBMCs were isolated from healthy controls and treated with a known ER stress inducer, Thapsigargin (TG), which activates UPR by inhibiting the ER Ca2+ ATPase. The basal expression levels of 3,746 probes were significantly changed in response to TG compared to untreated PBMCs. Analysis of enriched functional terms among the probes with increased and decreased expression showed terms generally associated with immune responses including B cell activation, cytokine production and inflammatory response, among others. Genes with increased expression were enriched in terms related to the endoplasmic reticulum and the unfolded protein response (**Table 2**). Genes with decreased expression showed significant enrichment in functional terms related to homeostasis, lysosome and GTPase signaling (**Table 3**). A complete functional enrichment data is available upon request.

Examination of specific sets of genes deregulated by TG treatment showed increased expression of heat shock proteins (HSP) and IFN-regulated genes **(Fig 3.2).** The HSP group consisted of HSP70 (HSP5A, HSP9B) and its co-chaperones, HSP40 isoforms (DNAJB1, DNAJB5, DNAJB11 and DNAJC3), HSP90 (HSPCB) and HSP150/110 (HSPH1). IFN related genes with altered expression included many well-known IFN-regulated genes: IFI44, IFI44L, IFIT1, IFIT2, IFIT3, IFIT5, IFITM3, MX1, IFN alpha–inducible proteins 2 and 3 (GlP2 and G1P3), Interferon-induced guanylate-binding protein 1 (GBP1), IFN-inducible RNA dependent protein kinase (PRKR), and IFN regulatory factor 4-1-7 (IRF4, IRF1, IRF7). Upregulation of HSP and representative

IFN-regulated genes in PBMCstreated with TG was confirmed by qPCR (data not shown).

Table 2. Functional terms enriched in probes with increased expression after TG treatment.

Term Name	Term	List	Genom	p-value
ER-nucleus signaling pathway	BP	35	98	1.01E-16
cellular response to unfolded protein	BP	32	84	4.21E-16
endoplasmic reticulum unfolded protein response	BP	32	84	4.21E-16
response to endoplasmic reticulum stress	BP	34	98	9.99E-16
response to unfolded protein	BP	40	143	5.08E-15
endoplasmic reticulum-Golgi intermediate compartment	CC	14	54	1.57E-03
endoplasmic reticulum-Golgi intermediate compartment membrane	CC	9	24	5.25E-03
melanosome	CC	19	108	1.07E-02
pigment granule	CC	19	108	1.07E-02
nucleosome	CC	18	105	2.74E-02
GTP binding	MF	47	399	9.96E-04
GTPase activity	MF	33	242	2.10E-03
guanyl nucleotide binding	MF	47	411	2.33E-03
guanyl ribonucleotide binding	MF	47	411	2.33E-03
sequence-specific DNA binding transcription factor activity	MF	90	997	2.46E-03
Protein processing in endoplasmic reticulum	KEGG	48	186	2.38E-14
Measles	KEGG	31	154	3.65E-06
NOD-like receptor signaling pathway	KEGG	16	65	4.82E-04
Protein export	KEGG	9	23	9.37E-04
Transcriptional misregulation in cancer	KEGG	29	185	1.97E-03
Cytosolic tRNA aminoacylation	E	13	27	4.04E-07
Insulin Synthesis and Secretion	E	14	46	1.03E-04
Translocation of Preproinsulin to Endoplasmic Reticulum	E	10	26	4.63E-04
tRNA Aminoacylation	E	13	49	1.54E-03
Amino acid transport across the plasma membrane	E	10	31	2.93E-03

Term Domain, functional annotation category of interest (see Materials and Methods for details). List total number of genes in the gene set annotated to a given term. Genome Total, number of genes in the genome annotated to a given term. P-value, measure of the significance of the functional enrichment corrected for multiple testing.

Table 3. Functional terms enriched in probes with decreased expression after TG treatment.

Term Name	Term Domain	List Total	Genome Total	p-value
wound healing	BP	97	651	4.89E-09
coagulation	BP	83	528	1.23E-08
hemostasis	BP	83	528	1.23E-08
blood coagulation	BP	82	524	2.10E-08
activation of immune response	BP	67	417	5.47E-07
lytic vacuole	CC	67	401	9.60E-08
lysosome	CC	67	401	9.60E-08
vacuole	CC	69	455	3.75E-06
actin cytoskeleton	CC	56	337	4.68E-06
ruffle	CC	23	117	1.55E-02
GTPase regulator activity	MF	81	445	6.77E-12
nucleoside-triphosphatase regulator activity	MF	82	457	1.06E-11
enzyme activator activity	MF	69	402	1.38E-08
small GTPase regulator activity	MF	55	294	6.75E-08
GTPase activator activity	MF	50	255	9.29E-08
Lysosome	KEGG	34	135	8.65E-08
Hematopoietic cell lineage	KEGG	25	108	8.45E-05
Leukocyte transendothelial migration	KEGG	25	115	2.89E-04
Fc gamma R-mediated phagocytosis	KEGG	22	97	5.44E-04
Regulation of actin cytoskeleton	KEGG	35	211	2.31E-03
Hemostasis	REACTOME	47	234	6.21E-08
Signaling in Immune system	REACTOME	62	431	7.30E-05
Signaling by Rho GTPases	REACTOME	26	117	1.39E-04
Rho GTPase cycle	REACTOME	26	117	1.39E-04
Formation of Platelet plug	REACTOME	24	118	2.12E-03

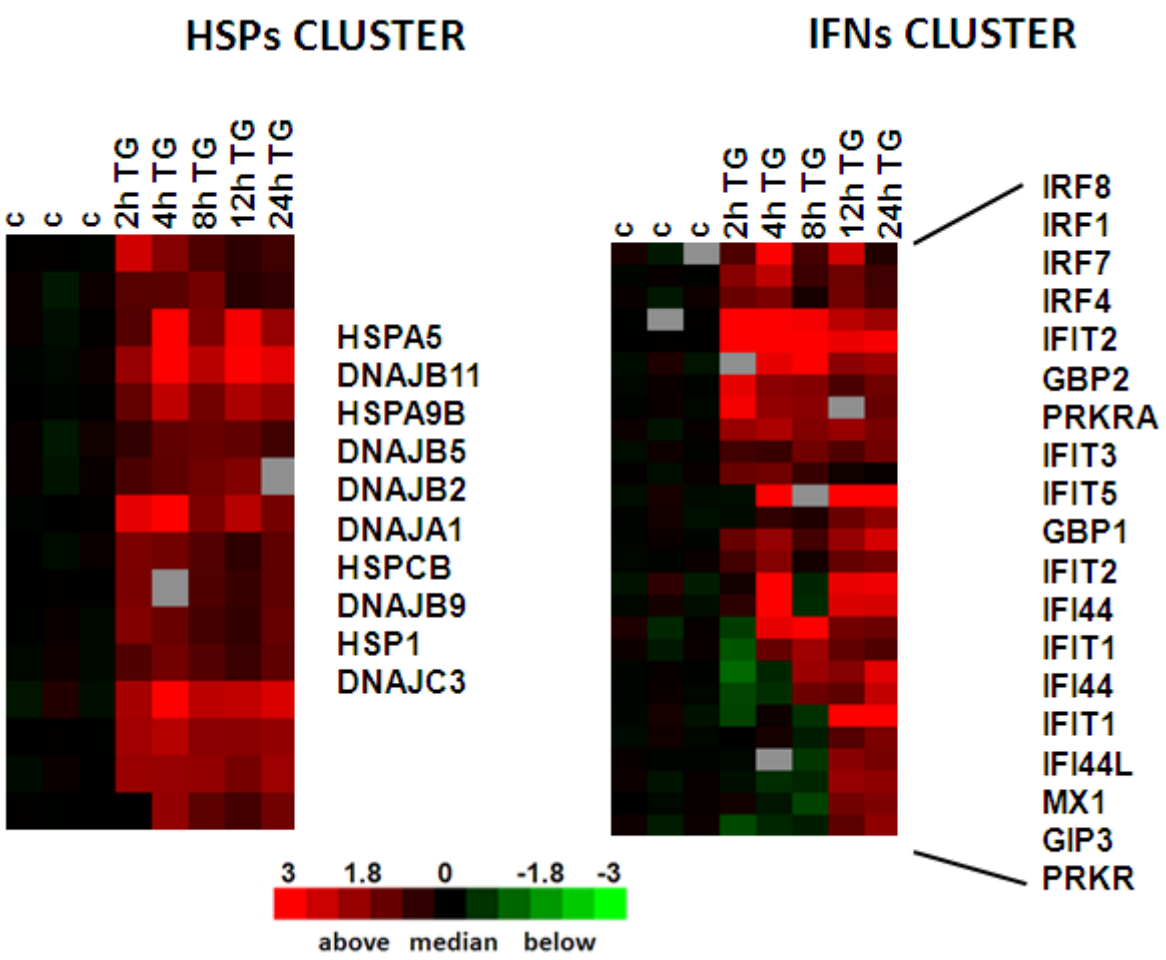

Figure 3.2. HSP and IFN-regulated gene expression in healthy control PBMCs treated with thapsigargin.

Heat map showing the expression of genes clustered using average linkage, hierarchical, and supervised clustering. Left, Cluster of HSP genes, right, Cluster of IFN-regulated genes. Values above the mean expression level of each gene are indicated in red and below the mean are indicated in green.

DNAJ/HSP40 genes are upregulated in PBMCs from lcSSc-PAH patients

DNAJ/HSP40 isoforms were among the genes most notably altered in the TG treated PBMCs and were subsequently analyzed in control and patient PBMCs by qRT-PCR. Compared with healthy control subjects, PBMCs from lcSSc-NoPAH patients demonstrated elevated expression of DNAJB1 ($p<0.0001$) with a further increase in lcSSc-PAH ($p<0.05$). DNAJB11 was less prominently, but also significantly increased in lcSSc-PAH samples ($p<0.05$), **(Fig. 3.3).** Furthermore, expression of DNAJB1 showed the highest positive correlation with pulmonary arterial pressure (PAP) by catheterization of lcSSc patients ($r=0.56$, $p< 0.05$). On the other hand, with the exception of BiP, expression of other HSP70 isoforms, as well as HSP90 and HSP150/110 isoforms was not significantly different between HC and lcSSc (with or without PAH) PBMCs (data no shown), suggesting a distinct association of HSP40 with lcSSc.

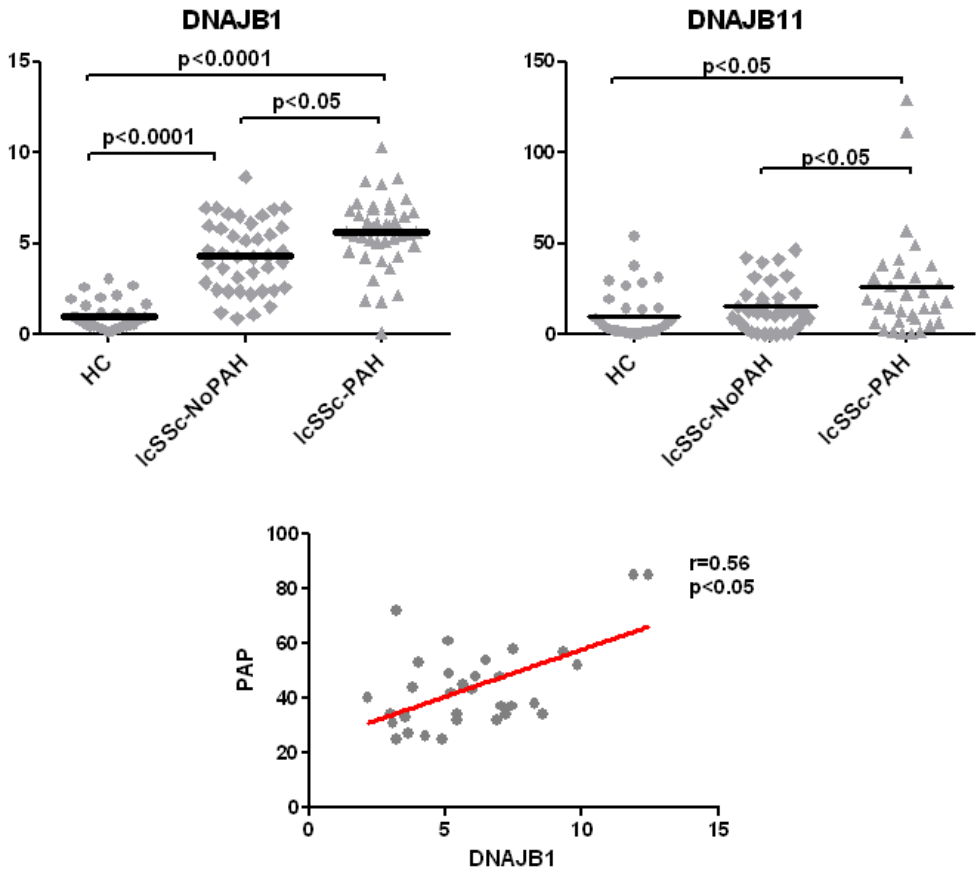

Figure 3.3. Elevated mRNA expression of selected DNAJ/HSP40 in patients with lcSSc.

PBMCs were isolated from HC (n=36), lcSSc-NoPAH (n=34) and lcSSc-PAH (n=32). mRNA levels of DNAJ/HSP40 (DNAJB1, DNAJB11) were measured by qPCR. Expression of the housekeeping gene β-actin served as an internal positive control. Data are expressed as the fold-change normalized to mRNA expression in HC samples. Each data point represents a single subject; horizontal lines show the mean. Bottom panel, linear regression analysis of the relationship between the mean pulmonary artery pressure (PAP) and DNAJB1 in PBMCs from lcSSc-PAH patients.

IFN-regulated genes are altered in PBMCs from lcSSc-PAH patients

The presence of "interferon signature" in SSc PBMCs, including patients with lcSSc has previously been reported (135, 136). From the cluster of the IFN-regulated genes induced by TG in HC PBMCs **(Fig. 3.2),** we selected several representative genes that were not previously validated in patient PBMCs, including IFIT1, IFIT2, IFITM1, and IRF4 for the qPCR analysis in PBMCs. Consistent with previous studies IFIT1, IFIT2, and IFITM1 were expressed at a significantly higher level in patients with lcSSc- NoPAH vs healthy controls **(Fig. 3.4)** but did not show a progressive increase in lcSSc-PAH patients compared to lcSSc-NoPAH. Interestingly, IRF4 was significantly downregulated in lcSSc vs HC (HC vs lcSSc-NoPAH, $p<0.0001$, HC vs lcSSc-PAH, $p<0.001$). Expression of other IRF genes, including IRF5, was not significantly changed.

To further investigate whether endogenously produced interferon was contributing to the UPRmediated upregulation of IFN-related genes, HC PBMCs were treated for 24 hours with TG (5pM) or IFNα (250U, used as control) in combination with B18R, a decoy receptor for type I Interferon. Selected IFN-induced genes were analyzed by qPCR. IFNα-induced IFIT1, IFITM1, MX1, CXCL10, IRF7 and IRF4 were abrogated by B18R. Although, blockade of IFN also attenuated upregulation of those genes in response to TG, the inhibitory effects were less potent, possibly suggesting contribution of the IFN-independent pathways (**Fig. 3.5).** Interestingly, while IRF4 was only moderately upregulated by IFNα, it was very potently upregulated by TG. Unexpectedly, B18R further potentiated stimulation of IRF4 by TG, suggesting that IFN may both positively and negatively regulate IRF4 expression depending on the activation status of other signaling pathways.

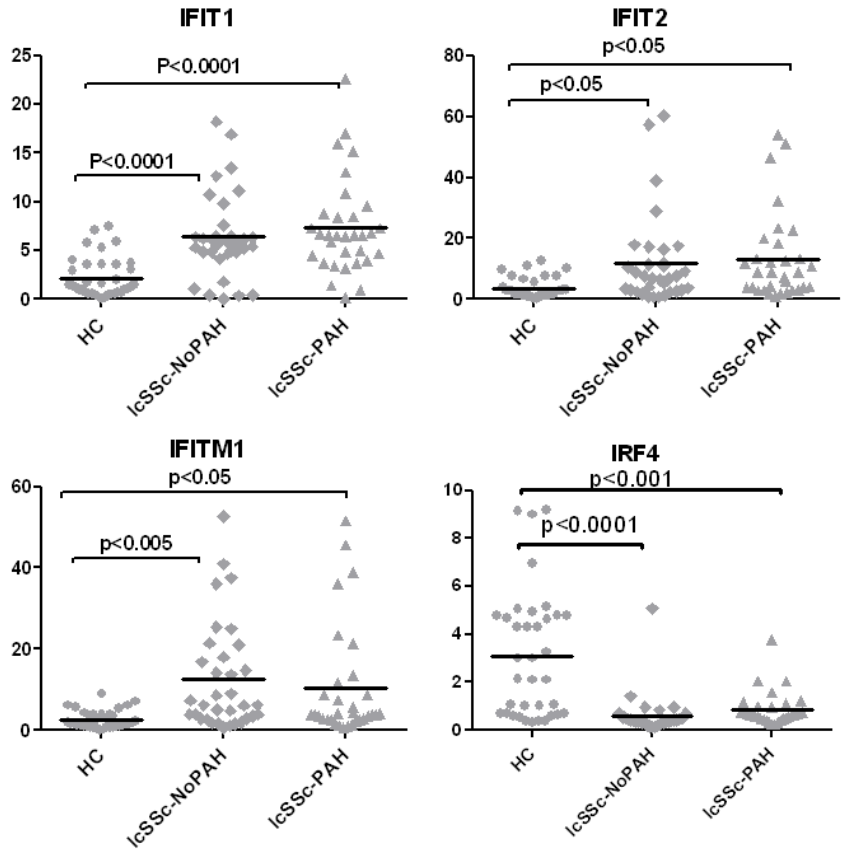

Figure 3.4. mRNA expression of IFN-regulated genes in patients with lcSSc.

PBMCs were isolated from HC (n=36), lcSSc-NoPAH (n=34) and lcSSc-PAH (n=32). mRNA levels of IFIT1, IFIT2, IFITM1 and IRF4 were measured by qPCR. Expression of the housekeeping gene β-actin served as an internal positive control. Data are expressed as the fold-change normalized to mRNA expression in HC samples. Each data point represents a single subject; horizontal lines show the mean.

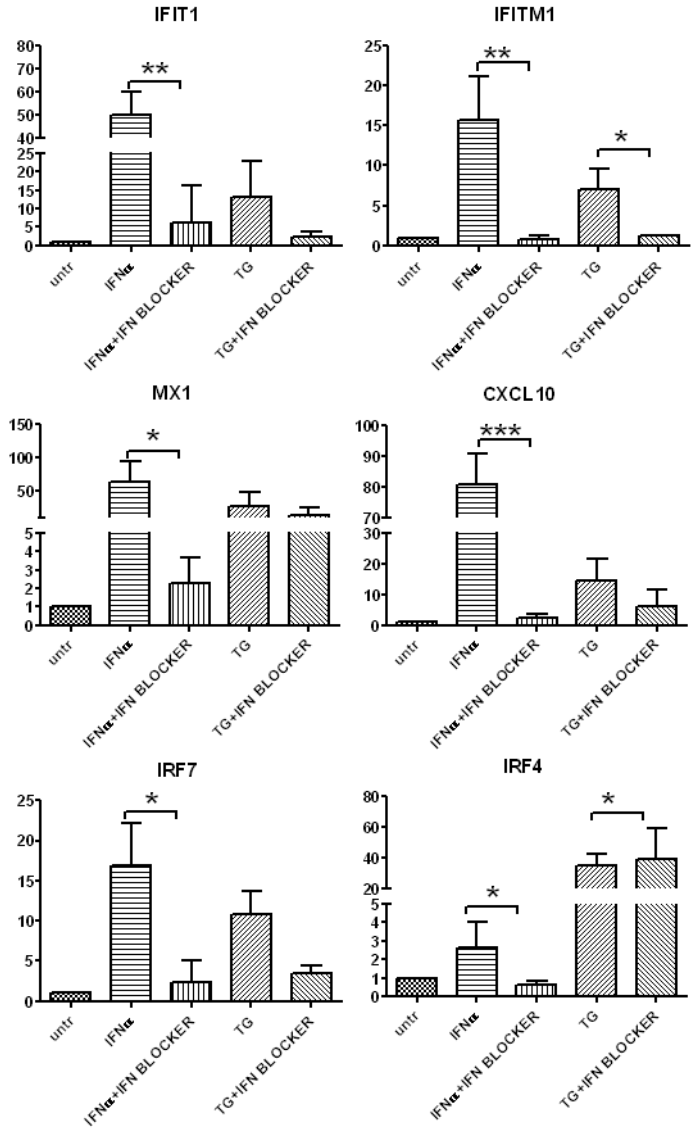

Figure 3.5. mRNA expression of IFN-regulated genes in HC PBMCs after IFN blocker treatment. PBMCs were isolated from HC and treated with IFNα or TG in combination with IFN blocker. mRNA levels of IFIT1, IFITM1, MX1, CXCL10, IRF7 and IRF4 were measured by qPCR. Expression of the housekeeping gene β-actin served as an internal positive control. Data are expressed as the fold-change normalized to mRNA expression in untreated HC PBMC.

ER stress/UPR correlates with increased expression levels of pro-inflammatory cytokines

Elevated expression levels of inflammatory mediators, including IL-6, IL-1β and IL-13 have been previously reported in PBMCs from lcSSc patients (137-139). mRNA expression of IL-6 was determined in PBMC samples used in this study. In agreement with previous findings IL-6 mRNA levels were significantly elevated in lcSSc vs healthy control ($p<0.005$) PBMCs, with the highest levels in lcSSc- PAH PBMCs. Notably, there was a positive correlation ($r = 0.53$, $p<0.0001$) between mRNA expression of IL-6 and BiP in PBMC samples from patients with lSSc **(Fig. 3.6).** Similar correlations were observed when mRNA level of DNAJB1 were compared with IL-6 levels (data not shown). These results suggest that ER stress/UPR could contribute to increased inflammation in patients with lcSSc.

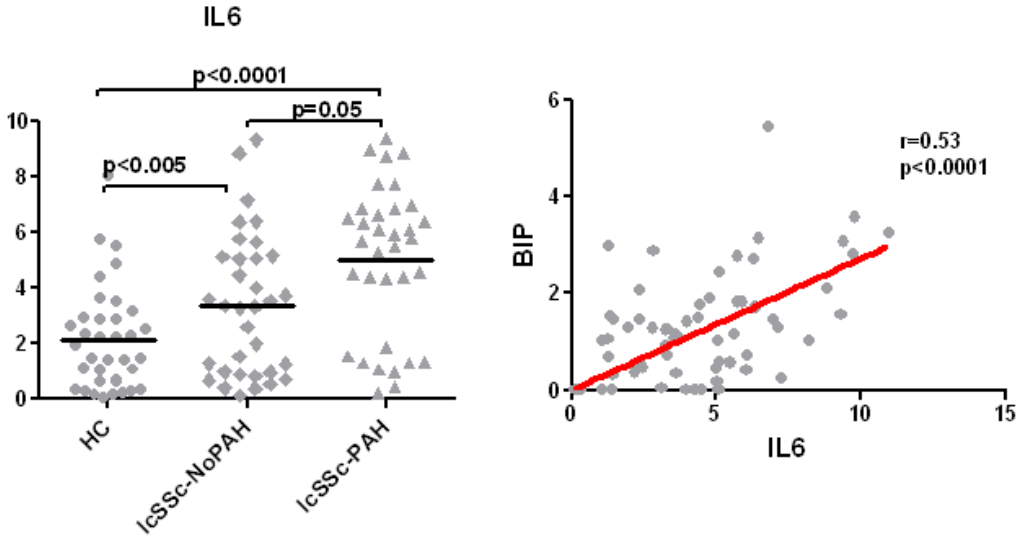

Figure 3.6. IL-6 expression correlates with ER stress/UPR genes.

mRNA levels of IL-6 was determined by qPCR in PBMC isolated from HC (n=36), lcSSc-NoPAH (n=34) and lcSSc-PAH (n=32). Expression of the housekeeping gene β-actin served as an internal positive control. Data are expressed as the fold-change normalized to mRNA expression in HC samples. Each data point represents a single subject; horizontal lines show the mean. Right panel depicts linear regression analysis of the relationship between expression of BiP and IL-6 in PBMCs from lcSSc patients.

DISCUSSION

This study characterized expression of ER stress and UPR related genes in PBMCs obtained from a well-characterized cohort of lcSSc patients with and without evidence of PAH, as well as healthy control subjects. We demonstrate that lcSSc PBMCs express significantly higher levels of genes representing all three branches of the UPR. Notably, BiP, ATF6, ATF4, as well as chaperone DNAJB1 showed the highest levels of expression in patients with PAH. On the other hand, spliced XBP1 was only moderately elevated in lcSSc patients with no evidence of further upregulation in patients with PAH. A less pronounced activation of the XBP1 pathway may indicate a chronic nature of the ER stress in patients with PAH (121). There was a significant correlation between expression of DNAJB1 and pulmonary arterial pressure, as well as BiP and IL-6 expression suggesting that chronic UPR may contribute to increased inflammation in lcSSc through activation of the NF-κB and AP-1 pathways (123).

The current study further expands the analyses of biomarkers in lcSSc PBMCs (136, 135). Similar to the previously observed pattern of gene expression in the "biomarker cluster" (IL3RA1, CCR1), selected ER chaperons, including BiP and DNAJB1, showed an increased expression in patients with lcSSc-NoPAH with the highest level of expression in patients with PAH. Expression of DNAJB, as well as BiP correlated strongly with PAP by right-heart catheterization. An earlier study by Christmann et al showed a strong association of the macrophage alternative activation marker gene, MRC1, with PAP, (136), but ER stress genes only weakly correlated with MRC1 ($r=0.32$, $p<0.05$ for MRC1 and BiP) and did not correlate with IL13RA1 but, instead correlated strongly with IL-6 (0.53, $p<0.0001$ for BiP and IL-6). These results

suggest heterogeneous mechanisms involved in worsening PAP in patients with lcSSc. Further studies are needed to determine whether increased levels of ER stress markers in lcSSc patients results from the changes in a specific subset of cells, e.g. monocytes or dendritic cells, or whether a majority of circulating cells contribute to the ER stress signature.

Growing evidence suggests that inflammation contributes to the development and/or progression of PAH. Plexiform lesions often contain lymphocytes, macrophages and mast cells, and antibody-complement deposits have been associated with PAH (43, 118). Furthermore, elevated expression levels of several inflammatory mediators have been observed in plasma of PAH patients (137). In particular, IL-6 has been implicated in the pathogenesis of PAH (136, 137) and increased levels of IL-6 have been reported in lcSSc-PAH patients (135, 138). Consistent with those earlier reports, we found elevated levels of IL-6 mRNA levels in lcSSc PBMCs, with the highest levels in lcSSc-PAH samples. Furthermore there was a positive and significant correlation between BiP and IL-6 mRNA expression levels, consistent with the possibility that ER stress may function as an endogenous inducer of inflammation. Interestingly, several of the ER stress/UPR genes showed variable expression in healthy individuals. A recent study by Dombroski et al (140) that characterized ER stress/UPR in human B cells obtained from healthy individuals have also found an extensive individual variability in expression of the ER stress/UPR related genes in response to the ER stress-inducing agents. Many of these variable genes were the known disease linked genes, suggesting a mechanistic link between ER function and human disease. Indeed, genetic polymorphisms in UPR genes have been functionally linked to inflammatory bowel

disease and asthma (141, 142). Relevant to these findings, we have previously shown that HLA-B35, which is associated with increased risk for developing PAH in Italian SSc patients (107, 103) induced ER stress-mediated upregulation of endothelin 1, proinflammatory cytokines, and IFNrelated genes in human microvascular endothelial cells (105). However, factors contributing to the induction of ER stress/UPR in patients with SSc are currently unknown.

Analyses of PBMCs subjected to thapsigargin-mediated ER stress *in vitro* revealed upregulation of a large number of IFN-regulated genes consistent with previous studies that demonstrated induction of IFNα by TG in mouse macrophages (RAW 264.7 cells) (143). Furthermore, priming of RAW 264.7 cells with TG greatly potentiated their responses to TLR4 and TLR3 ligands and this synergistic interaction was completely dependent on XBP-1 (143). In agreement with the previous reports (136, 138, 139), we have also observed increased expression of IFN-related genes, including IFIT1, IFIT2, and IFITM1 that correlated with increased XBP-1 splicing in our lcSSc patient cohort, suggesting that ER stress may contribute, among other factors, to the "interferon signature" in lcSSc patients. Consistent with this conclusion, we found that upregulation of IFN-regulated genes by TG in PBMCs was in part dependent on activation of the endogenous type I IFN. Unexpectedly, while both IFNα and TG induced IRF4 in HC PBMCs, there was a significant decrease of IRF4 expression in lcSSc-NoPAH and lcSSc- PAH PBMCs compared to healthy individuals, suggesting that ER stress and/or IFN-dependent pathways may not be involved in the downregulation of IRF4 in lcSSc patients. Given the prominent role of IRF4 in regulation of the immune response, in the future it would be important to focus on the mechanisms involved in its

dysregulation in lcSSc. IRF4 is expressed in both T and B cells and plays an important role in mature CD4+ T cell function (rev. in (144). Interestingly, a lack of IRF4 results in enhanced IFNα production under TH2 conditions (144). Additional studies revealed that peritoneal macrophages isolated from *Irf4*-deficient mice showed markedly enhanced induction of IL-6 and IL- 12p40 in response TLR4, TLR7 or TLR9 stimulation (145). Functionally, IRF4 was shown to compete with IRF5, but not IRF7 for MyD88 interaction (145). Since IRF5 expression levels were not changed in lcSSc PBMCs analyzed in this study (data not shown), lower levels of IRF4 may alter IRF5 signaling in this group of patients. Importantly, an IRF5 polymorphism was identified as one of the susceptibility loci associated with SSc in a recent SSc-genome wide association study (146). Together, these studies suggest that low expression levels of IRF4 may contribute to the enhanced cytokine production in lcSSc patients.

In conclusion, we demonstrate increased expression of selected UPR genes in PBMCs from patients with lcSSc. Since activation of UPR may result from a variety of factors that disturb cellular homeostasis, additional studies will be required to investigate specific factors contributing to this process in lcSSc. It may be relevant that viral infections have been associated with induction of UPR, as well as the interferon response (147), and since herpesviruses have been linked to SSc pathogenesis (148-150), it may suggest a possible role for a herpesvirus in activation of the UPR response in lcSSc. Regardless of the initial stimulus, activation of the UPR may signal disease worsening and selected ER stress/UPR genes may serve as markers of disease progression in patients with lcSSc. Interestingly, activation of ER stress/UPR markers

was recently demonstrated in monocytes from patients with type 2 diabetes and was implicated in attenuation of the response to TLR agonists (151). It may also be relevant that an altered TLR-mediated induction of proinflammatory cytokines was observed in dendritic cells from patients with SSc, including lcSSc (152). Future studies should determine whether ER stress/UPR alter the function of immune cells in patients with lcSSc.

Chapter 4

Role of the presence of HLA-B35 in activation of immune cells

Specific Aim 2.3. To determine whether HLA-B35 contributes to activation of ER stress in immune cells

Our previous study in ECs showed that the presence of HLA-B35 leads to changes in the expression of genes associated with ER stress and furthermore, activation of UPR pathway correlated with upregulation of interferon-regulated genes and other inflammatory genes (JI paper). The presence of ER stress and UPR markers in endothelial cells expressing HLA-B35 has suggested that HLA-B35 could induce ER stress in other cell types, including immune cells.

We decided to determine whether HLA-B35 influences the expression of ER stress/UPR genes in PBMC of patients with lcSSc-PAH. To explore the potential contribution of HLA-B35 to immune aspects of the disease, we stratified all PBMC samples based on the presence of the HLA-B35 allele.

The presence of HLA-B35 allele exacerbates activation of only selected ER stress/UPR in PBMCs

Among the previously tested ER stress markers, only BiP was consistently higher in PBMCs HLA-B35 positive compared with HLA-B35 negative samples, with statistically significant differences ($p<0.05$) observed in each lcSSc subset, as well as in healthy control samples **(fig.4.1).** ATF4 showed significant differences in lcSSc-noPAH subset and in healthy control samples ($p<0.005$ HC B35- vs B35+ and $p<0.001$ lcSSc-noPAH B35- vs B35+) but not in lcSScPAH samples. Furthermore, the elevated levels of ATF6 observed in lcSSc-PAH samples, were not influenced by the presence of HLA-B35 antigen. In contrast lcSSc-PAH HLA-B35-positive individuals showed a significantly

higher expression of spliced XBP1 compared to lcSSc HLA-B35-negative patients (p<0.05)(**fig.4.1).**

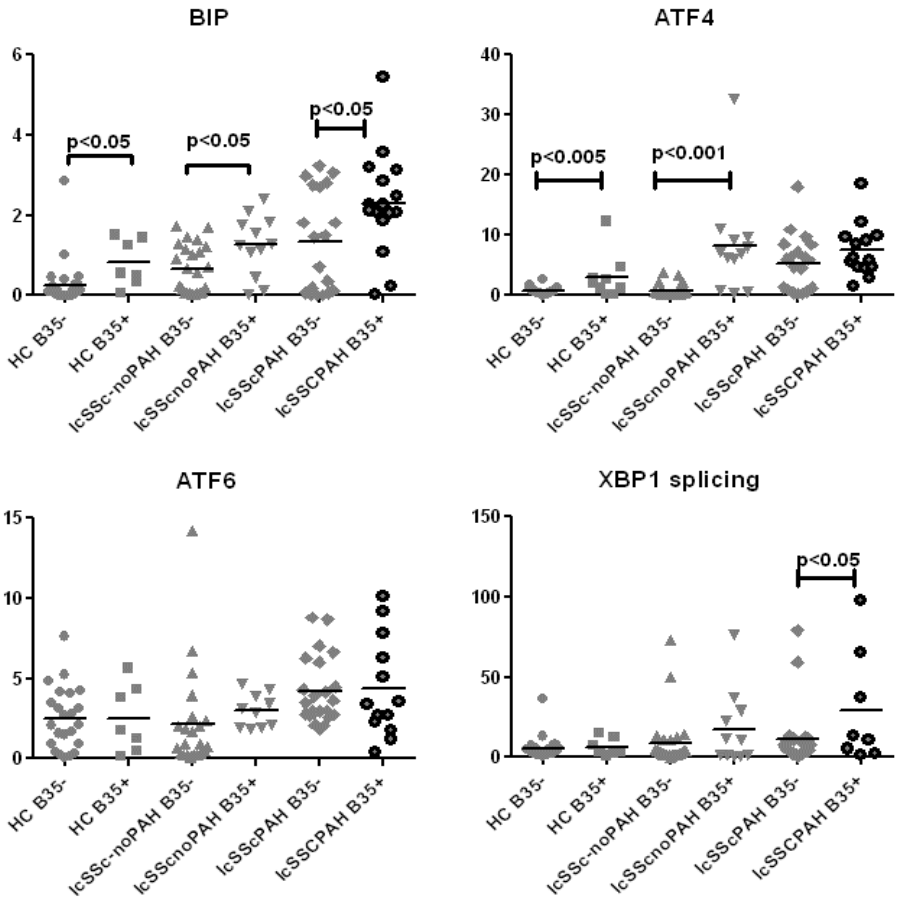

Figure 4.1. HLA-B35 correlates with higher expression of selected ER stress/UPR genes.

PBMCs were isolated from HC (n=36), lcSSc-NoPAH (n=34) and lcSSc-PAH (n=32) and grouped according to the presence of the HLA-B35 allele: HC B35+ (n = 7), HC B35- (n=29); lcSSc-NoPAH B35+ (n = 12), lcSSc-NoPAH B35- (n=22); lcSSc-PAH B35+ (n = 14), lcSSc-PAH B35- (n=18). mRNA levels of BiP and ATF4 (top panel), ATF6 and ATF6 (bottom panel). mRNA were measured by qPCR. Expression of the housekeeping gene β-actin served as an internal positive control. Data are expressed as the fold-change normalized to mRNA expression in a single HC sample. Each data point represents a single subject; horizontal lines show the mean.

Interestingly, the presence of HLA-B35 showed positive correlation with DNAJB1, with statistically significant differences observed in each group ($p<0.0001$ HC B35- vs HC B35+, $p< 0.05$ lcSSc-NoPAH B35+ vs lcSsc-NoPAH B35- and lcSSc-PAH B35+ vs lcSsc-PAH B35-) **(Fig.4.2).** The correlation between DNAJB1 and severity of PAH (PAP) previously observed ($r= 0.56$) was not related to presence of antigen HLA-B35 in the PBMC patients.

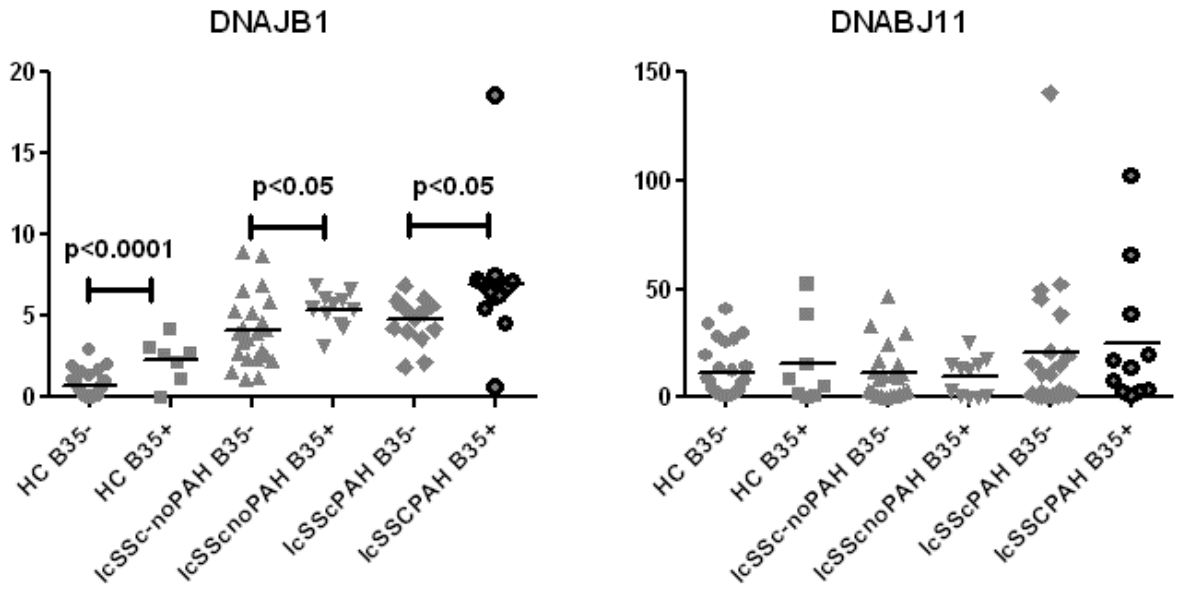

Figure 4.2. HLA-B35 correlates with higher expression of selected HSP40/DNAJB

PBMCs were isolated from HC (n=36), lcSSc-NoPAH (n=34) and lcSSc-PAH (n=32) and grouped according to the presence of the HLA-B35 allele: HC B35+ (n = 7), HC B35- (n=29); lcSSc-NoPAH B35+ (n = 12), lcSSc-NoPAH B35- (n=22); lcSSc-PAH B35+ (n = 14), lcSSc-PAH B35- (n=18). mRNA levels of DNAJB1 and ADNAJB11. mRNA were measured by qPCR. Expression of the housekeeping gene β-actin served as an internal positive control. Data are expressed as the fold-change normalized to mRNA expression in HC sample. Each data point represents a single subject; horizontal lines show the mean.

The presence of HLA-B35 allele in PBMC enhances the correlation between ER stress/UPR and increased expression levels of IL-6

With regard to IFN-regulated genes, when patients were stratified based on the presence of the HLA-B35 allele, we could observed a significantly higher level of IFIT1 expression in lcSSc samples ($p<0.05$ NoPAH B35- vs NoPAH B35+ and PAH B35- vs PAH B35+). Interestingly, healthy control HLA-B35 positive samples showed lower level of IRF4 compared to HC HLA-B35 negative samples. On the other hand, we observed a slightly higher level of IRF4 in HLA-B35 positive lcSScPAH patients ($p<0.05$) **(fig.4.3).**

As shown before, IL-6 mRNA levels were significantly elevated in lSSc vs healthy control ($p<0.0001$) PBMCs, with the highest levels in lcSSc-PAH PBMCs. When PBMCs were stratified based on the presence of the HLA-B35 allele, the expression level of IL-6 was consistently higher in HLA-B35 positive lcSSc PBMCs **(fig.4.4).** Statistically significant differences between HLA-B35-positive and HLA-B35-negative samples were observed in lcSSc-NoPAH (B35+ vs B35-, $p<0.05$) and lcSSc-PAH (B35+ vs B35-, p,0.05) but not in healthy controls. Previously we showed a positive correlation ($r = 0.53$, $p< 0.0001$) between mRNA expression of IL-6 and BiP in PBMC samples from patients with lcSSc. Notably, IL-6 expression was also correlated with the presence of HLA-B35. When lcSSc samples were stratified for HLA-B35 positive and HLA-B35 negative, the correlation between IL6 and BiP in B35+ samples was higher than the HLA-B35 negative samples ($r=0.38$ vs r+0.16).

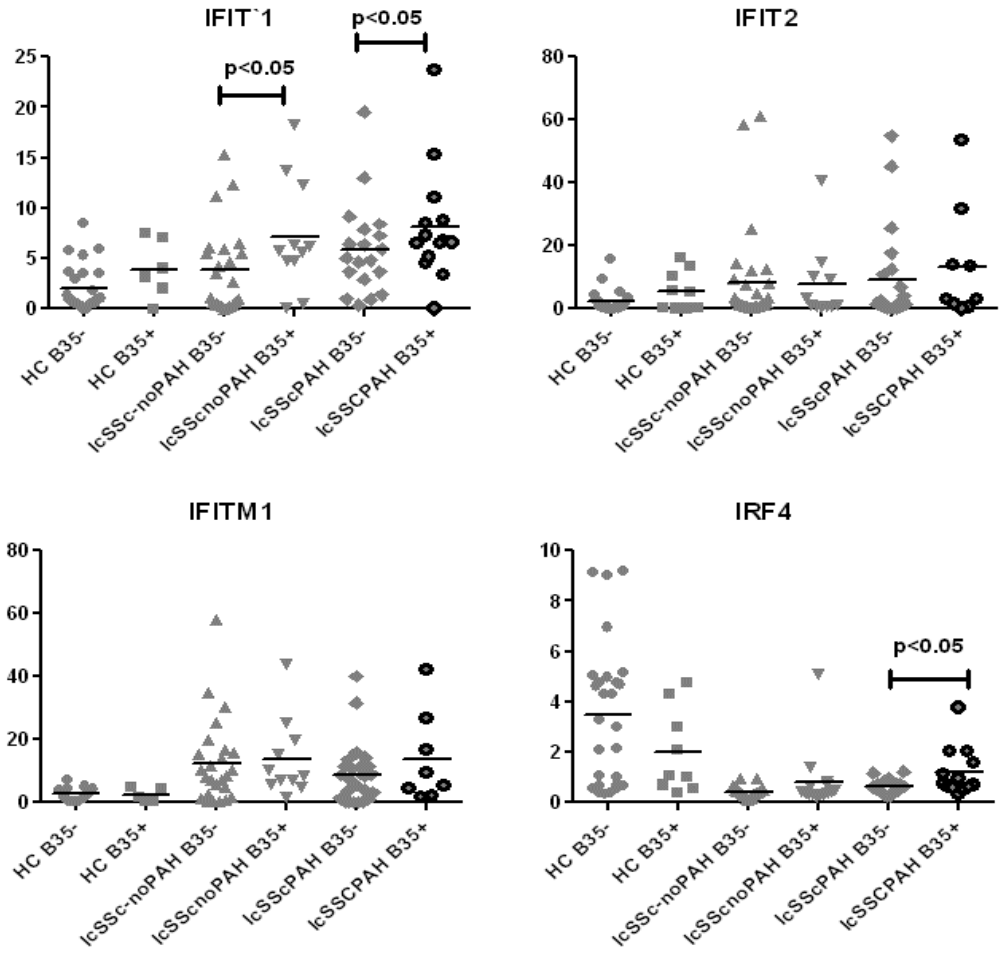

Figure 4.3. HLA-B35 correlates with expression of some Type I IFN induced genes.

PBMCs were isolated from HC (n=36), lcSSc-NoPAH (n=34) and lcSSc-PAH (n=32) and grouped according to the presence of the HLA-B35 allele: HC B35+ (n = 7), HC B35- (n=29); lcSSc-NoPAH B35+ (n = 12), lcSSc-NoPAH B35- (n=22); lcSSc-PAH B35+ (n = 14), lcSSc-PAH B35- (n=18). mRNA levels of IFIT1, IFIT2, IFITM1 and IRF4 were determined by. Expression of the housekeeping gene β-actin served as an internal positive control. Data are expressed as the fold-change normalized to mRNA expression in HC sample. Each data point represents a single subject; horizontal lines show the mean. Right panels represent MX1 and IFIT1 mRNA levels in PBMCs stratified based on the presence of the HLA-B35 allele.

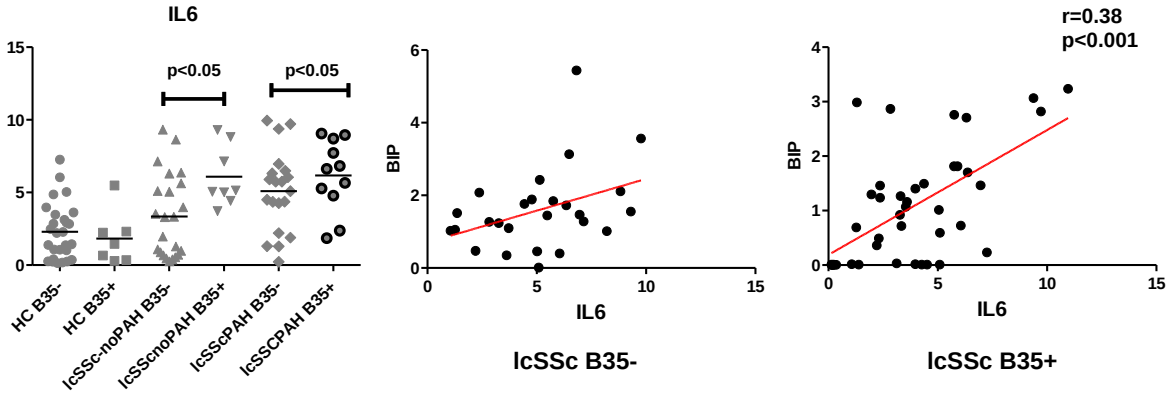

Figure 4.4. IL-6 expression correlates with ER stress/UPR genes.

PBMCs were isolated from HC (n=36), lcSSc-NoPAH (n=34) and lcSSc-PAH (n=32) and grouped according to the presence of the HLA-B35 allele: HC B35+ (n = 7), HC B35- (n=29); lcSSc-NoPAH B35+ (n = 12), lcSSc-NoPAH B35- (n=22); lcSSc-PAH B35+ (n = 14), lcSSc-PAH B35- (n=18). mRNA levels of IL-6 was determined by qPCR. Expression of the housekeeping gene β-actin served as an internal positive control. Data are expressed as the fold-change normalized to mRNA expression in a single HC sample. Each data point represents a single subject; horizontal lines show the mean. Left panel depicts linear regression analysis of the relationship between expression of BiP and IL-6 in PBMCs from lcSSc B35 negative and B35 positive patients.

Conclusion

Our analyses of PBMCs from HLA-B35 positive individuals showed higher levels of selected ER stress markers when compared to HLA-B35 negative individuals. In particular, cellular chaperones, BiP and DNAJB1 showed higher levels of expression in both healthy controls and lcSSc patients. This is agreement with our previous study linking HLA-B35 to stimulation of ER stress in microvascular endothelial cells. Together, our published and preliminary results support the view that genetic factors could contribute to the increased levels of ER stress at least in a subset of lcSSc patients.

We found elevated levels of IL-6 mRNA. We also observed a positive and significant correlation between BiP and IL-6 mRNA expression levels in HLA-B35 positive patients. These results support the theory that HLA-B35 could have a direct role in inducing IL-6 expression, possibly through the activation of ER stress/UPR pathways.

In conclusion we hypothesized that activation of ER stress/UPR, in combination with genetic factors, might drive the inflammatory process in SSc-PAH.

Further studies are needed to determine a specific “HLA-B35 signature” in lcSSc PBMCs. An array analyses on HC PBMC transduced with lentvirus carrying HLA-B35 will help us to determine specific ER stress gene upregulated that will be tested on our patient samples.

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Chapter 5

DISCUSSION

Discussion

PAH is a severe disease with worse survival rates for patients with lcSSc-PAH, who have a poorer response to therapy. All forms of PAH are characterized by severe pulmonary vascular remodeling that leads to increased vascular resistance and ultimately right heart failure. The molecular mechanisms underlying the remodeling process still remains elusive despite recent major strides made in the understanding of the pathogenesis of PAH.

Certain conditions are associated with PAH, such as congenital heart disease or connective tissue disease, but in the absence of these associated conditions, the causes of PAH remain a mystery. . A role for genetic association has been recognized supported by familial clustering patterns and high prevalence of the disease among certain ethnic groups. (distribution between female and male patients, age of onset, and natural history) (153,154).

The discovery of heterozygous mutations of the *BMPR2* gene, encoding for the bone morphogenetic protein receptor-II (BMPR-II), in a substantial proportion of patients with familial pulmonary arterial hypertension (FPAH), as well as many cases of sporadic or idiopathic disease (IPAH), represents perhaps the single greatest advance toward an understanding of the molecular mechanisms that underpin this puzzling and often lethal vascular disease (155-159). The overall result is a deficiency in functional BMPR-II but it is not clear yet how loss-of-function mutations are actually related to the development of this disease.

We believe that understanding the mechanism of genetic predisposition to PAH is essential to the discovery of the root pathogenesis.

Various ethnic populations of SSc patients also exhibit specific expression patterns of human leukocyte antigens (HLA) and autoantibodies. Several reports suggest that HLA class I alleles, besides their pivotal role in antigen presentation, can modulate cell signaling (160-167). Furthermore, our previous studies showed that expression of HLA-B35 at the physiological level found in B35-positive individuals influences the production of the two key regulatory molecules, ET-1 and eNOS, involved in maintaining vascular homeostasis. The presence of HLA-B35 significantly increased endothelin-1, while at the same time significantly decreased endothelial nitric oxide synthase production (107), thus strongly suggesting that HLA-B35 could play pathogenic role in PAH by directly contributing to vasoconstriction. However, the mechanisms underlying this association have not been fully elucidated yet.

In an effort to identify genes differentially expressed in dermal microvascular ECs, a microarray analysis revealed high upregulation of Heat Shock Proteins (HSPs) in ECs expressing HLA-B35. In particular HSP-70 (HSPA1A and HSPA1B) and its co-chaperone, HSP40 (DnaJB1 and DNAJB9) were upregulated suggesting the activation of ER stress and unfolded protein response (UPR) in cells expressing HLA-B35.

Accumulating evidence indicates that ER stress is associated with a range of diseases, including neurological disorders, diabetes, metabolic disease, intestinal inflammation and autoimmunity making ER stress a probable instigator of pathological cell death and dysfunction (161-164). Interestingly, there is also evidence that ER

homeostasis is closely related to regulation of inflammatory gene expression. A link between ER stress and the inflammatory response was reported in different experimental models, including endothelial cells and immune cells (162, 165-167). Inflammation can be triggered by chronic excess of metabolic factors, cytokines, and hormones, and those factors can also trigger ER stress, which can further disrupt metabolic function, leading to more inflammation (167, 168).

The association between ER stress response and expression of the HLA allele was previously observed for HLA-B27, a central mediator of many spondyloarthropathies (SpA), including ankylosing spondylitis (169). While the mechanism whereby HLA-B27 contributes to development of SpA is complex and not fully understood, one of the proposed pathogenic events involves protein misfolding. Several studies have shown that HLA-B27 heavy chain (HC) exhibits abnormal properties, including a tendency to misfold and to accumulate in the ER thereby triggering an ER stress response and activation of the UPR (170-173). Since the effects of HLA-B35 observed in our *in vitro* cell model point to an internal mechanism, it is conceivable that by the analogy with HLA-B27, pathogenic role of HLA-B35 could also be related to slow or improper HC folding.

All these observations suggested us the possibility that HLA-B35 could induce ER stress and UPR response resulting in deregulated expression of ET-1 and indicating a possible pathogenic role of HLA-B35 in PAH disease.

The goal of my research was to determine a pathogenic role of HLA-B35/ER stress in lcSSc-PAH by contributing to endothelial cell dysfunction, as well as to activation of immune system in lcSSc-PAH patients.

Endothelial cells constitute a first line of defense protecting tissues from injury. Elevated production of ET-1 is a common characteristic associated with endothelial cell dysfunction in various pathological conditions, including pulmonary arterial hypertension. Previous studies have shown that HLA-B35 is associated with an increased risk for developing PAH in patients with scleroderma (SSc) (83).

In this study we show for the first time that ATF4, a mediator of ER stress, is a novel regulator of the ET-1 gene in endothelial cells. ATF4 contributes to the basal expression of ET-1 and is required for the induction of ET-1 in response to HLA-B35/ER stress. Our results strongly suggest that activation of the eIF2α/ATF4 pathway leads to increased formation of the ATF4 protein complexes with c-JUN which activates ET-1 transcription through the AP1 response element.

The current study further supports the potential pathogenic role of HLA-B35/ER stress in upregulating ET-1 production and may clarifies in part the molecular mechanism involved in this process in endothelial cells supporting a key role for the eIF2α-ATF4 pathway in response to vascular injury (112)..

We also expanded the analyses of biomarkers in lcSSc PBMCs (135-139). Selected ER chaperons, including BiP and DNAJB1, showed an increased expression in patients with lcSSc-NoPAH with the highest level of expression in patients with PAH.

Expression of DNAJB, as well as BiP correlated strongly with PAP by right-heart catheterization.

Growing evidence suggests that inflammation contributes to the development and/or progression of PAH. Elevated expression levels of several inflammatory mediators have been observed in plasma of PAH patients (137). In particular, IL-6 has been implicated in the pathogenesis of PAH (137,138) and increased levels of IL-6 have been reported in lcSSc-PAH patients (136, 138). Consistent with those earlier reports, we found elevated levels of IL-6 mRNA levels in lcSSc PBMCs, with the highest levels in lcSSc-PAH samples. Also there was a positive and significant correlation between ER stress (BiP) and IL-6 mRNA expression levels, consistent with the possibility that ER stress may function as an endogenous inducer of inflammation.

Furthermore, genetic polymorphisms in UPR genes have been functionally linked to inflammatory diseases (141, 142). Relevant to these findings, we have previously shown that HLA-B35, which is associated with increased risk for developing PAH in Italian lcSSc patients (83, 107), induced ER stress-mediated upregulation of ET-1, proinflammatory cytokines, and IFN-related genes in human microvascular endothelial cells (107).

In agreement with our previous study linking HLA-B35 to stimulation of ER stress in microvascular endothelial cells, HLA-B35 positive individuals showed slightly higher levels of some ER stress markers when compared to HLA-B35 negative individuals. Notably, higher levels of some ER stress/UPR markers were also present in PBMCs obtained from healthy individuals carrying the HLA-B35 allele, supporting the view that

genetic factors could contribute to the increased levels of ER stress at least in a restricted population of SSc patients. Our observation is consistent with an another study that showed ER stress/UPR activation in human B cells obtained from healthy individuals is consistent with this notion (140). This suggested that there is a genetically determined extensive individual variability in expression of ER stress/UPR related genes in response to ER stress-inducing agents. Many of these variable genes are known disease-linked genes, suggesting a mechanistic link between ER function and human disease.

In conclusion we demonstrated a novel paradigm that activation of ER stress/UPR pathways is the key process contributing to the pathogenesis of lcSSc-PAH (see diagram **fig. 5.1**). Although, ER stress/UPR has previously been linked to autoimmunity 1, metabolic diseases 2 and intestinal inflammation 3, these pathways have not been investigated in the context of lcSSc. Our studies correlate the degree of ER stress/UPR with the clinical status of lcSSc patients and provide new knowledge on the pathogenic effects of HLA-B35, providing the first understanding of functional genomics in lcSSc-PAH

However, factors contributing to the induction of ER stress/UPR in patients with lcSSc-PAH are currently unknown but selected ER stress/UPR genes may serve as markers of disease progression in patients with lcSSc.

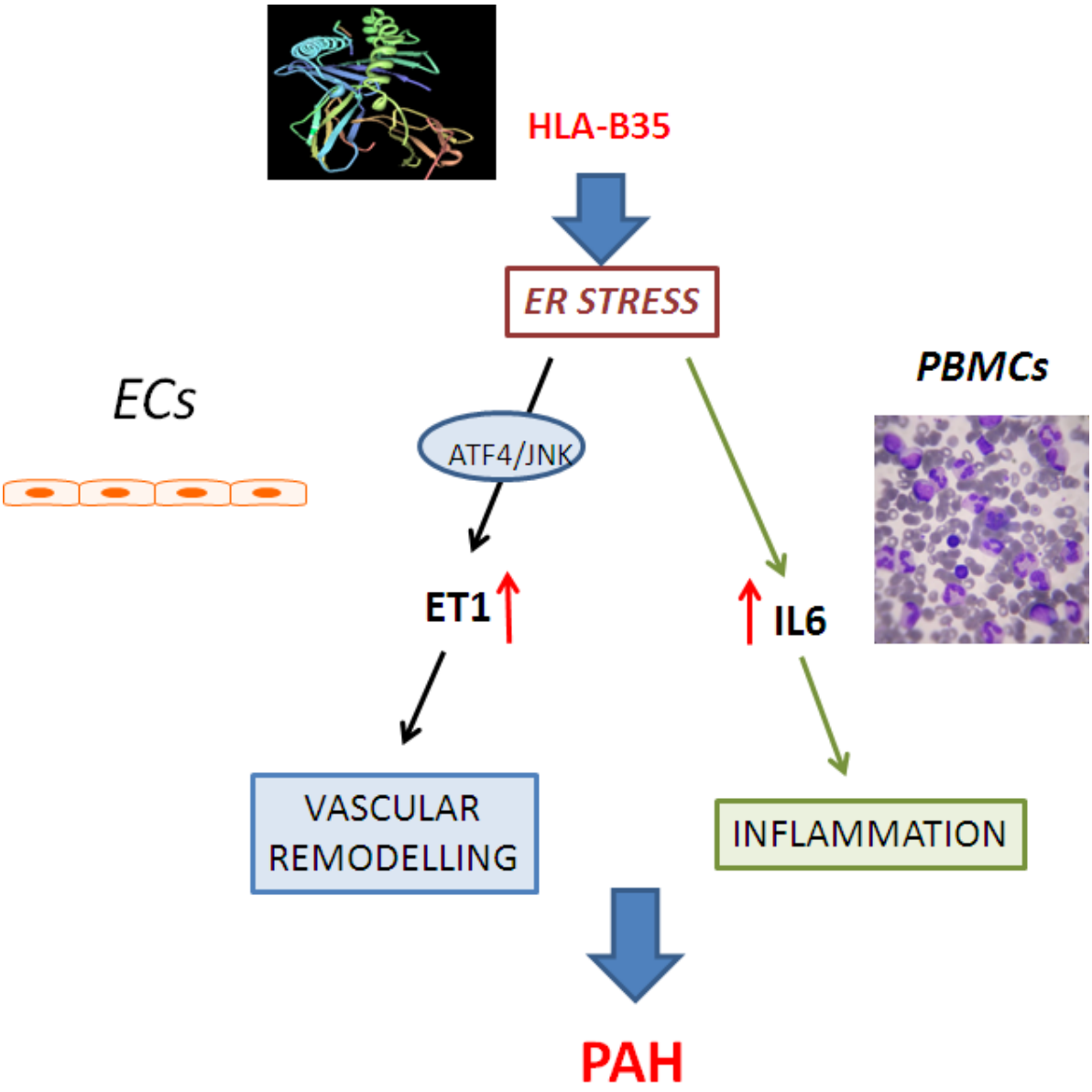

Figure 5.1. Schematic diagram showing hypothetical role of HLA-B35/ER in the pathogenesis of lcSSc-PAH.

In endothelial cells, HLA-B35 induced upregulation of ET1 via activation of ATF4 suggesting a potential pathogenic role of HLA-B35/ER in vascular injury. In PBMC, HLA-B35/ER stress induced increased of IL6 expression suggesting that chronic UPR may contribute to increased inflammation.

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