

HLA-B35 Upregulates Endothelin-1 and Downregulates Endothelial Nitric Oxide Synthase via Endoplasmic Reticulum Stress Response in Endothelial Cells

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The presence of the HLA-B35 allele has emerged as an important risk factor for the development of isolated pulmonary hypertension in patients with scleroderma, however the mechanisms underlying this association have not been fully elucidated. The goal of our study was to determine the molecular mechanisms that mediate the biological effects of HLA-B35 in endothelial cells (ECs). Our data demonstrate that HLA-B35 expression at physiological levels via adenoviral vector resulted in significantly increased endothelin-1 (ET-1) and a significantly decreased endothelial NO synthase (eNOS), mRNA, and protein levels. 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 endoplasmic reticulum (ER) stress and unfolded protein response in ECs. Examination of selected mediators of the unfolded protein response, including H chain binding protein (BiP; GRP78), C/Ebp homologous protein (CHOP; GADD153), endoplasmic reticulum oxidase, and protein disulfide isomerase has revealed a consistent increase of BiP expression levels. Accordingly, thapsigargin, a known ER stress inducer, stimulated ET-1 mRNA and protein levels in ECs. This study suggests that HLA-B35 could contribute to EC dysfunction via ER stress-mediated induction of ET-1 in patients with pulmonary hypertension. *The Journal of Immunology*, 2010, 184: 4654–4661.

The HLA system genes are members of the MHC in humans that consists of >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 Ags are involved in the presentation of peptides, predominantly derived from intracellular proteins, to CD8⁺ cytotoxic T cells. HLAs class II Ags are functionally specialized for presentation of short protein fragments (antigenic peptides), mainly derived from extracellular proteins, to the TCR on CD4⁺ Th cells. HLA genes are highly polymorphic and this 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 Ags and susceptibility to diseases (1, 2) or progression of infectious diseases

(3–9). However, these differences do not explain all the associations between HLA and diseases.

Several reports suggest that HLA class I alleles, besides their pivotal role in Ag presentation, can act as signal transducing molecules that influence individual reactivity to external stimuli (10–17). Furthermore previous studies showed that HLA class I molecules differ in their ability to modulate cell signaling, suggesting the existence of a haplotype-specific regulation of signal transduction (18–22). More recently, it has been suggested that the increased susceptibility to apoptosis of HLA-B35 expressing cells, especially in B35/B35 homozygotes, underlies the well-known association between this Ag and the rapid progression of HIV infection toward AIDS with opportunistic infections (23–27). Apoptosis is known to be an important factor in causing lymphocyte depletion in acquired AIDS patients (28) and this process is further enhanced by the class I overexpression that is induced in tissues during viral infections. Our previous studies suggested that the upregulation of endothelin-1 (ET-1) in activated HLA-B35–positive endothelial cells (ECs) (29) may be the basis of the association between HLA-B35 allele and the isolated pulmonary hypertension (iPHT) in Italian scleroderma (SSc) patients (30, 31). However, the mechanisms underlying this association have not been fully elucidated.

PHT is a complex, multifactorial disease involving numerous biochemical pathways and different cell types leading to alterations in vascular reactivity, vascular structure, and interactions of the vessel wall with circulating blood elements. 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 microvessels, causing fixed alterations in pulmonary resistance (32). The normal pulmonary endothelium maintains a low vascular resistance, suppresses inflammation, vascular smooth muscle growth, inhibits platelet adherence, and aggregation. In patients with PHT, the endothelium lose these vasoprotective functions (33, 34). The PHT endothelium is characterized by the reduced production of vasodilators, such as NO

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Abbreviations used in this paper: Ad, adenovirus; ATF, activating transcription factor; BiP, H chain binding protein; CHOP, C/Ebp homologous protein; EC, endothelial cell; eNOS, endothelial NO synthase; ER, endoplasmic reticulum; ERO1, endoplasmic reticulum oxidase; ET-1, endothelin-1; HDMEC, human dermal microvascular endothelial cell; HSP, heat shock protein; iPHT, isolated pulmonary hypertension; PDI, protein disulfide isomerase; PPET1, preproendothelin-1; TG, thapsigargin; UPR, unfolded protein response; SSc, scleroderma.

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and prostacyclin, and increased elaboration of vasoconstrictors, mitogens, and prothrombotic and proinflammatory mediators, such as thromboxane, ET, plasminogen activator inhibitor, and 5-lipoxygenase. This imbalance between vasoconstricting and vasodilating mediators contributes significantly to the pathology of PHT (35–37).

ET-1, a cytokine produced by activated ECs, has been implicated as the main pathogenic molecule in the development of SSc-associated PHT. Increased levels of ET-1 are found in sera from SSc patients with iPHT (29, 38–42). Excess of ET-1 is associated with dramatic structural changes in the pathology of PHT vasculature, including inflammation, vasoconstriction, cell proliferation, and fibrosis. Previously published studies demonstrated that ET-1 production is influenced by the presence of the HLA-B35 allele in ECV304 cell line (29), suggesting a role for HLA-B35 in activating ET-1 gene expression. In this study, we have focused our investigation on further delineation of this intriguing phenomenon. The results demonstrate that expression of HLA-B35 at the physiological level found in HLA-B35-positive individuals induces changes in the expression of genes related to endoplasmic reticulum (ER) stress in ECs. This study suggests that this pathway may contribute to the development of PHT in SSc patients.

Materials and Methods

Reagents

Thapsigargin (TG) was purchased by Sigma-Aldrich (St. Louis, MO). Tissue culture reagents, M199 and 100× antibiotic antimycotic solution (penicillin, streptomycin, and amphotericin B) were purchased by Life Technologies BRL (Grand Island, NY), EBM kit by Lonza (and FBS by HyClone [Logan, UT]). Enhanced chemiluminescence reagent and BCA protein assay reagent were obtained from Pierce Chemical (Rockford, IL).

Abs used were as followed: goat ET-1 Ab (Santa Cruz Biotechnology, Santa Clara, CA) at a 1:500 dilution; endothelial NO synthase (eNOS) Ab (Santa Cruz Biotechnology) at a 1:1000 dilution; heat shock protein 70 (HSP70) and HSP40 Ab (Cell Signaling, Danvers, MA) at 1:1000 dilution; H chain binding protein (BiP) and C/Ebp homologous protein (CHOP) Ab (Abcam, Cambridge, MA) at 1:1000 dilution; monoclonal β -actin Ab (Sigma-Aldrich) at 1:5000 dilution.

Cell culture

ECV304 cell lines were purchased from the European Cell Culture Collection. The cells were grown in M199 medium supplemented with 10% FBS and EC growth supplement in an incubator with humidity atmosphere and 5% CO₂ at 37°C. HUVECs and human dermal microvascular ECs (HDMECs) were purchased from Lonza Walkersville (Walkersville, MD). 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. HUVECs and HDMECs harvested between passage 2 and 6 were used for experiments.

Adenoviral constructs

An adenoviral vector expressing HLA-B35 (or B8) was generated using the method described by He et al. (43). Briefly, the cDNA encoding HLA-B35/B8 was cloned in the shuttle vector pAdTRACK-CMV, which contains a GFP expression cassette driven by a separate CMV promoter, and was used to generate recombinant adenoviruses (Ads). An Ad expressing GFP alone was generated via the same method for use as a control vector. The dose used to transduce ECs were 5–10 multiplicities of infection (MOIs) of the Ad (dose of Ad that expresses HLA-B35 at the physiological levels corresponding to those found in HLA-B35⁺ individuals). 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 analysis or for Western blot.

Microarrays

Total RNA from cell cultures was extracted using the Qiagen RNeasy kit (Valencia, CA). The RNA quality and yield were assessed by a Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) and a NanoDrop

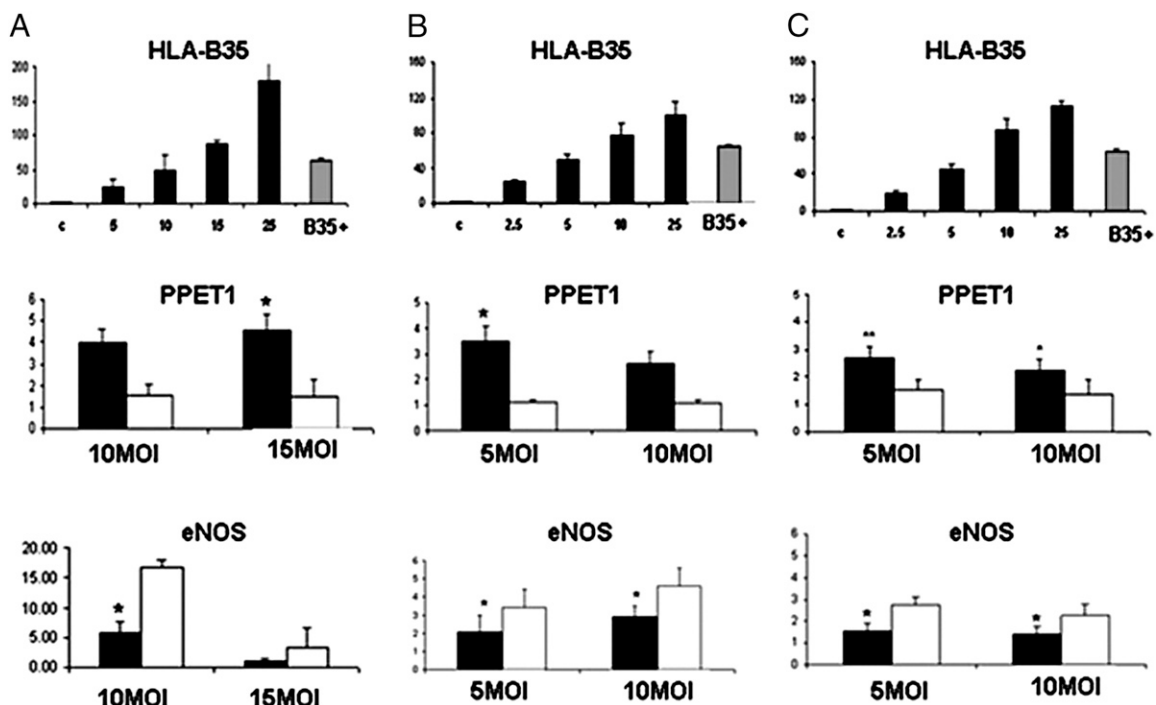


FIGURE 1. Upregulation of PPET1 and downregulation of eNOS mRNA after HLA-B35 (and HLA-B8) over-expression in ECV304 (A), HUVEC (B), and HDMEC (C) cells. In the *top panel*, we show the dose of Ad that expresses HLA-B35 at the physiological levels corresponding to those found in HLA-B35⁺ individual (gray column) in ECV304 (A), HUVECs (B), and HDMECs (C). Confluent dishes of ECs were transduced with 10 and 15 MOI (ECV304) or 5 and 10 MOI (HUVECs and HDMECs) of Ad encoding HLA-B35 and HLA-B8 for 48 h. Total RNA was extracted and mRNA levels of PPET1 (*middle row*) and eNOS (*bottom row*) 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. * p = 0.05; ** p = 0.001 Ad-B35/GFP (black column) versus Ad-B8/GFP (white column).

ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Two hundred nanograms of total RNA was amplified and purified using IlluminaTotalPrep RNA Amplification Kit (Applied Biosystems/Ambion, Austin, TX) according to the vendor's instructions. The amplified cRNA was hybridized on Illumina Human Ref-8 v2 arrays, and the data were extracted with IlluminaBeadstudio software suite (Illumina, San Diego, CA).

Complete microarray data were deposited in GEO public database (www.ncbi.nlm.nih.gov/geo/, accession number GSE20055).

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 are listed in Table I.

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, 1 mM 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 Na_3VO_4) 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 TBS washes, membranes were probed with HRP-conjugated secondary Ab against the appropriate species for 1–2 h at roomtemperature. Protein levels were visualized using ECL reagents (Amersham Biosciences, Piscataway, NJ).

Results

HLA-B35 modulates ET-1 and eNOS mRNA and protein expression in ECs

The effects of HLA-B35 and HLA-B8 (another Ag of class I, not known to be associated with an increased risk for developing iPHT in patients with SSc) were studied in primary ECs. For these studies, we generated Ad vectors expressing *HLA-B* alleles and a GFP protein under control of a separate CMV promoter (Ad-B35/GFP and Ad-B8/GFP). In a dose-titration experiment, we established a dose of Ad that results in HLA-B35 expression at the physiological levels corresponding to those found in HLA-B35⁺ individual (gray column, Fig. 1, top row).

Based on the previously reported observation that showed stimulatory effect of the *HLA-B35* allele on ET-1 production in ECV304 cell line, we first confirmed the effects of Ad-B35/GFP and Ad-B8/GFP on expression of endothelin-1 gene (preproendothelin-1 [PPET1]) in ECV304 (Fig. 1A, middle panel; see Table I for primers). We next investigated the effects of HLA-B35 in HUVECs and HDMECs. As shown in Fig. 1B, 1C (middle row) Ad-B35/GFP stimulated PPET1 mRNA in primary ECs. Specifically, PPET1 mRNA level was increased 3.5-fold \pm 0.58, $p = 0.05$ in HUVEC cells transduced with 5 MOI of Ad-B35/GFP (Fig. 1B, middle row) and 2.7-fold \pm 0.4, $p = 0.001$ and 2.25-fold \pm 0.4, $p = 0.05$ in HDMEC cells transduced with 5 and 10 MOI of Ad, respectively (Fig. 1C, middle row).

Table I. Primers sequences for quantitative PCR

Primer	Sequence
HLA-B35	Forward 5'-gaccggaacacacagatctt-3' Reverse 5'-ggaggaggcggccctcg-3'
HLA-B8	Forward 5'-gaccggaacacacagatctt-3' Reverse 5'-ccgcgcctcagcgtg-3'
PPET-1	Forward 5'-gctcgtccctgatgataaa-3' Reverse 5'-ccatacggaaacagtct-3'
NOS3	Forward 5'-aggaacctgtgacctca-3' Reverse 5'-tatccaggtccatgcagaca-3'
HSPA1A	Forward 5'-gaagaaggtgctggacaagt-3' Reverse 5'-gatgggttacacacctgt-3'
HSPA1B	Forward 5'-aggccagcaagatccat-3' Reverse 5'-cgtctccctgttctactt-3'
DNAJB1	Forward 5'-aggcaagccccacaatct-3' Reverse 5'-agagtgggacgttctct-3'
DNAB9	Forward 5'-ccaccctgacaaaataagagc-3' Reverse 5'-cgtctattagcatctgagatgtttc-3'
BiP (GRP94)	Forward 5'-aagaagctattcagttggatga-3' Reverse 5'-ttctgtaactcgcttga-3'
CHOP (GADD153)	Forward 5'-ctgaatcgcaccaagcatga-3' Reverse 5'-ttctgtaactcgcttga-3'
ERO1	Forward 5'-taaacctgaagaggcgtgt-3' Reverse 5'-tgacatggttgacagaca-3'
PDI	Forward 5'-tgaccagtggaataaaaa-3' Reverse 5'-tccttccctgtatcaatt-3'
β -actin	Forward 5'-aatgtcgcggaggaccttgattgc-3' Reverse 5'-aggatggcaaggactctcttaa-3'

Because PHT is characterized by a decreased production of endogenous NO, we next examined whether HLA-B35 over-expression modulates eNOS expression. We observed that eNOS

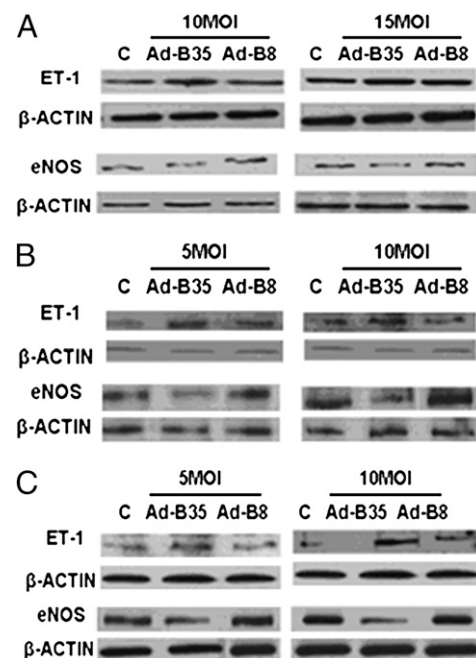


FIGURE 2. Expression of ET-1 and eNOS protein levels in ECV304 (A), HUVEC (B), and HDMEC (C) cells transduced with HLA-B35 or HLA-B8 Ads. ECs were transduced with 10 and 15 MOI (ECV304) or 5 and 10 MOI (HUVECs and HDMECs) of Ad-B35/GFP or Ad-B8/GFP for 48 h; 20 μ g of total cellular proteins were separated via 15% SDS-PAGE for ET-1 (7.5% for eNOS) and 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 re-probed for β -actin using a mAb to β -actin. Representative blots of at least three experiments are shown.

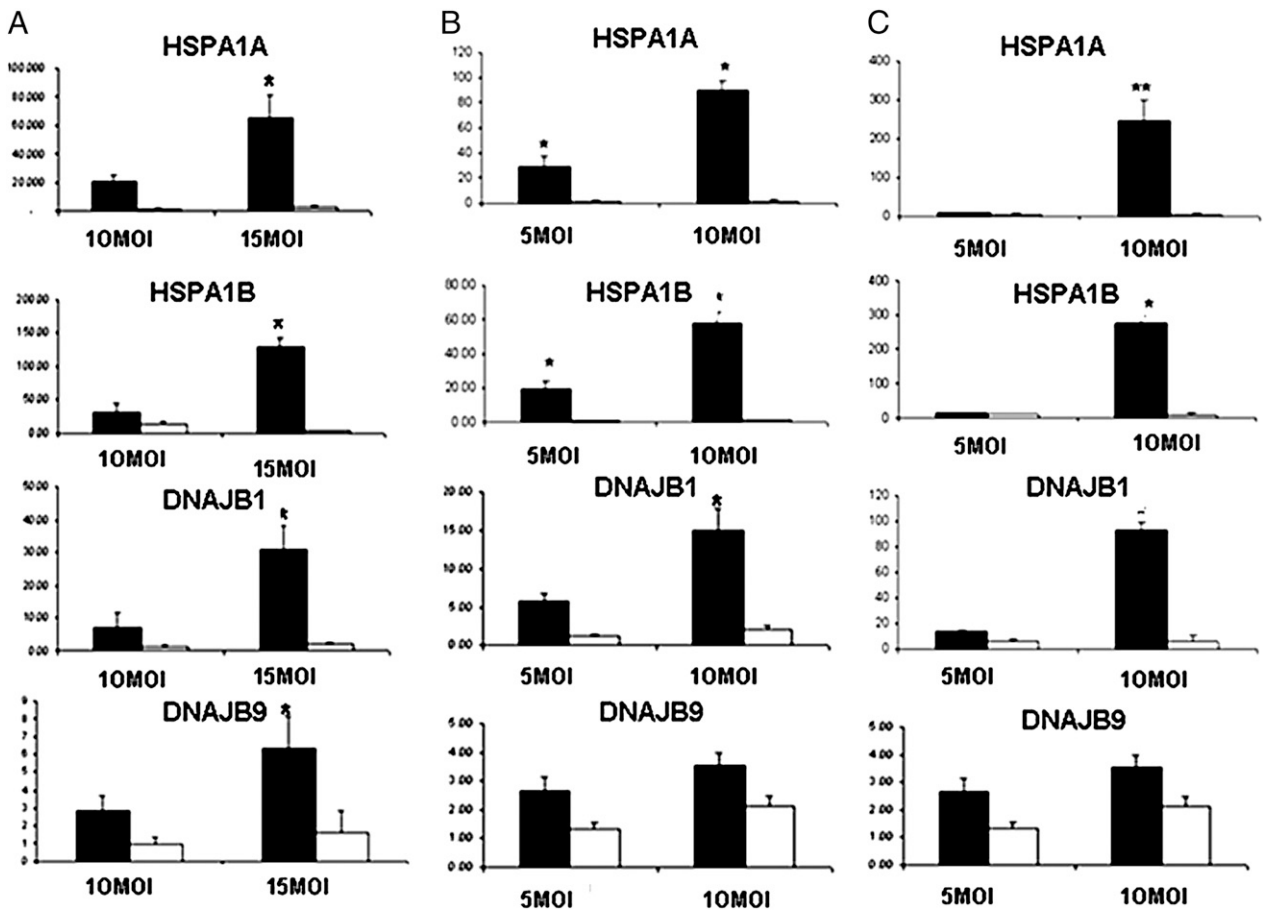


FIGURE 3. Expression of HSPs in ECV304 (A), HUVEC (B), and HDMEC (C) cells transduced with HLA-B35 (HLA-B8) Ads. Total RNA was isolated from ECs transduced with 10 and 15 MOI (ECV304) or 5 and 10 MOI (HUVECs and HDMECs) of Ad-B35/GFP or Ad-B8/GFP for 48 h. Quantitative RT-PCR was performed with SYBR Green and β -actin as an internal control. * p = 0.05; ** p = 0.001 Ad-B35/GFP (black column) versus Ad-B8/GFP (white column).

mRNA levels were significantly decreased in all three cell types transduced with Ad-B35/GFP as compared with cells transduced with Ad-B8/GFP (Fig. 1A–C, bottom row). This observation was extended to proteins expression.

Consistent with mRNA measurements ET-1 protein levels were increased and eNOS protein levels were reduced in cells expressing HLA-B35/GFP (Fig. 2). However, ET-1 and eNOS were expressed at the similar levels in cells transduced with Ad-B8/GFP and control virus.

HLA-B35 upregulates HSPs in ECs

Microarrays analyses was used to further investigate the effects of HLA-B35 on gene expression in ECs. The basal expression levels of 418 genes were significantly changed in cells transduced with Ad-B35/GFP as compared with cells transduced with Ad-B8/GFP. Among the highly upregulated genes were HSP-70 (HSPA1A and -1B) and its co-chaperone, HSP40 (DNAJB1 and -B9) (see Supplemental Table I for additional genes).

We verified the mRNA expression levels of the HSPs by real-time PCR (Fig. 3) in ECV304 (Fig. 3A), HUVECs (Fig. 3B), and HDMECs (Fig. 3C) transduced with Ad-B35/GFP or Ad-B8/GFP. Both isoforms of HSPA (1A and 1B) and DNAJ (B1 and -B9) were markedly upregulated in the presence of HLA-B35. Consistent with mRNA data, HLA-B35 markedly increased levels of HSP70 and HSP40 proteins, particularly in primary cell lines (HUVECs and HDMECs) (Fig. 4). There were no significant differences in HSP70 protein expression between control and cells transduced with Ad-B8/GFP.

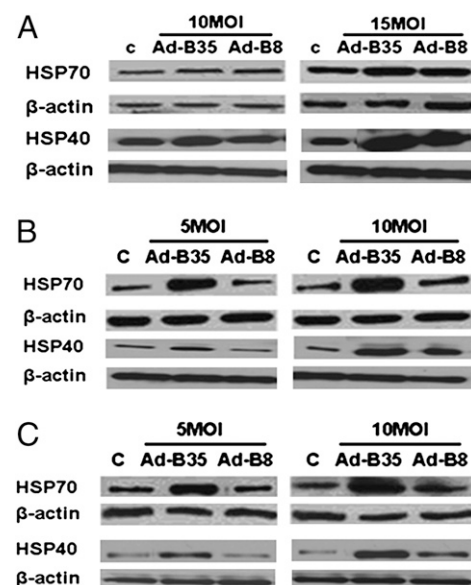


FIGURE 4. Expression of HSP70 and HSP40 protein levels in ECV304 (A), HUVEC (B), and HDMEC (C) cells transduced with HLA-B35 (HLA-B8) Ads. The 30 μ g total cellular proteins were separated via 10% SDS-PAGE and transferred to a nitrocellulose membrane. The blots were probed overnight with 1:1000 dilutions of primary Abs in 3% milk/Tween-Tris buffered saline at 4°C. As a control for equal protein loading, membranes were stripped and reprobed for β -actin using a mAb to β -actin. Representative blots of at least three experiments are shown.

HLA-B35 overexpression induces unfolded protein response

The upregulation of HSPs by HLA-B35 has suggested the possibility that HLA-B35 induces ER stress and unfolded protein response (UPR). Therefore we examined the status of selected mediators of UPR: BiP, CHOP, endoplasmic reticulum oxidase (ERO1), and protein disulfide isomerase (PDI). Only BiP, an ER resident protein considered to be the master regulator of UPR, was significantly upregulated by Ad-B35/GFP at the mRNA level (Fig. 5). Likewise, a protein level of BiP was increased by HLA-B35 in all cell types (Fig. 6). The increase was more pronounced at the higher MOI of the Ad. Interestingly, protein levels of CHOP were modestly increased by the treatment with HLA-B8 when compared with the cells treated with HLA-B35 (Fig. 6).

TG treatment induces ET-1 mRNA and protein expression

To test the notion that ER stress contributes to the activation of ET-1 gene expression, we examined the effect of TG on the ET-1 mRNA and protein levels. ECV304 cells (Fig. 7, left panel) and HDMECs (Fig. 7, right panel) were treated for 24 h with several doses of TG (1–10 pM), which moderately increased the expression levels of HSPA1A (Fig. 7A). Treatment with TG consistently increased ET-1 mRNA (Fig. 7B, top row) and protein levels (Fig. 7B, bottom row) with the maximal increase observed with 5 pM TG. Higher levels of TG were toxic to the cells. On the other hand, the effect of TG

on eNOS expression levels was inconsistent with both down-regulation and upregulation observed in the individual experiments (data not shown).

Discussion

In this study, we present evidence 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 ET-1, whereas in the same time significantly decreasing eNOS production, thus strongly suggesting that HLA-B35 could play pathogenic role in PHT by directly contributing to vasoconstriction. In addition, our data demonstrates that HLA-B35 has pathogenic effect that extends beyond modulation of vascular tone. We observed that changes in the ET-1 and eNOS levels correlated with the significant upregulation in expression of several HSPs, including HSPA1A and -1B, DNAJB1 and -B9. HSPs are a group of proteins present in all cells, whose expression is increased when the cells are exposed to stress condition. These proteins are “chaperones” that assist a large variety of folding processes, ranging from folding of newly synthesized proteins to facilitation of proteolytic degradation of unstable proteins. The cochaperones (HSP40) determine the activity of HSP70s by stabilizing their interaction with substrate proteins because ATP hydrolysis is essential for their activity (44–47). The

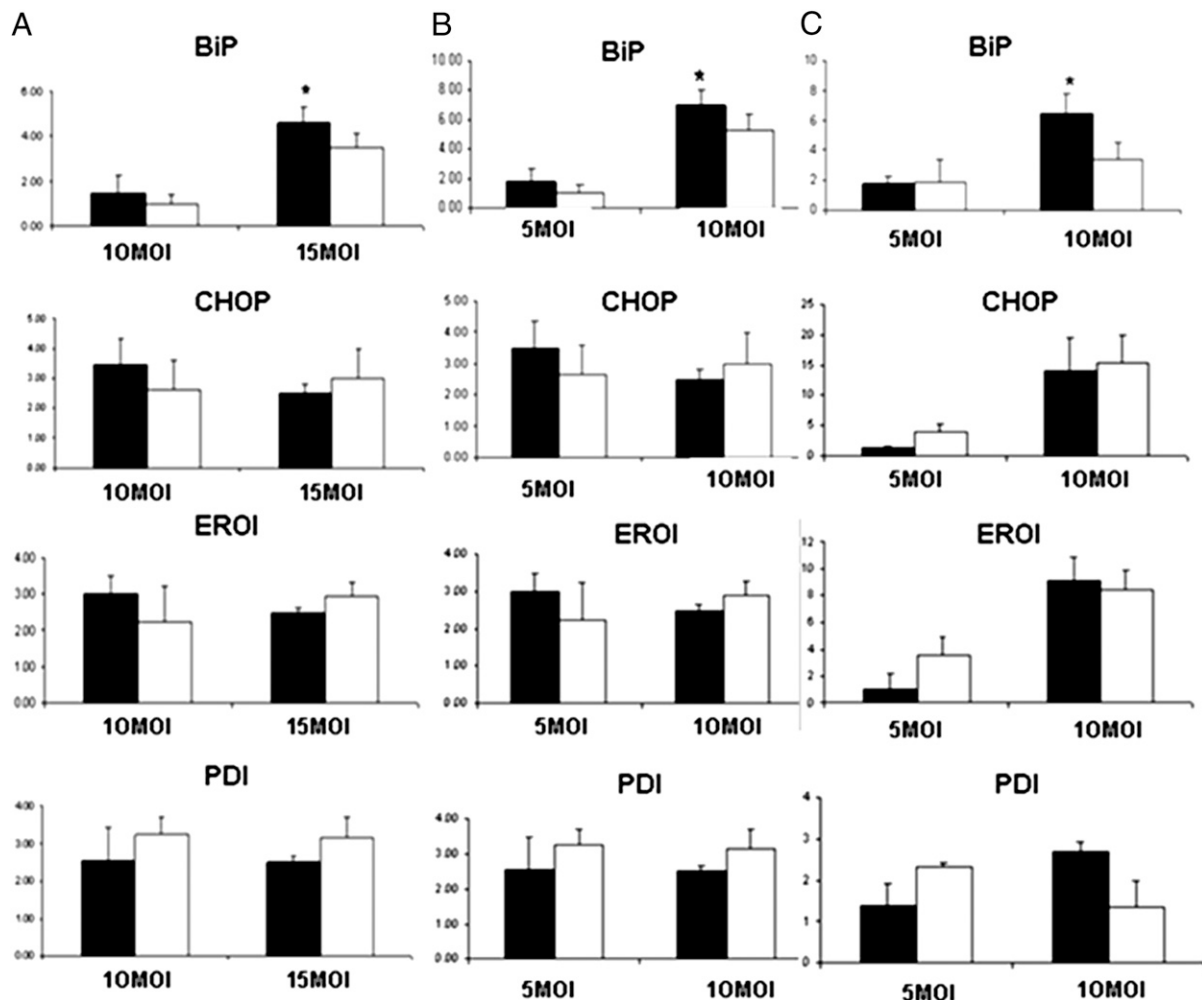


FIGURE 5. Expression of UPR genes in ECV304 (A), HUVEC (B), and HDMEC (C) cells transduced with HLA-B35 (HLA-B8) Ads. Total RNA was isolated from ECs transduced with 10–15 (ECV304) or 5–10 (HUVECs and HDMECs) MOI of Ad-B35/GFP (Ad-B8/GFP) after 48 h. Quantitative RT-PCR was performed with SYBR Green and β -actin as an internal control. * $p = 0.05$ Ad-B35/GFP (black column) versus Ad-B8/GFP (white column).

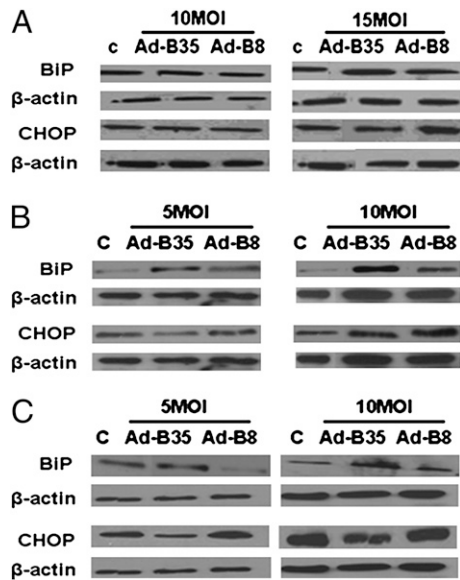


FIGURE 6. Expression of BiP and CHOP protein levels in ECV304 (A), HUVEC (B), and HDMEC (C) cells transduced with HLA-B35 (HLA-B8) Ads. The 30 μg total cellular proteins were separated via 10% SDS-PAGE and transferred to a nitrocellulose membrane. The blots were probed overnight with 1:1000 dilutions of primary Abs in 3% milk/Tween-Tris buffered saline at 4°C. As a control for equal protein loading, membranes were stripped and reprobed for β-actin using a mAb to β-actin. Representative blots of at least three experiments are shown.

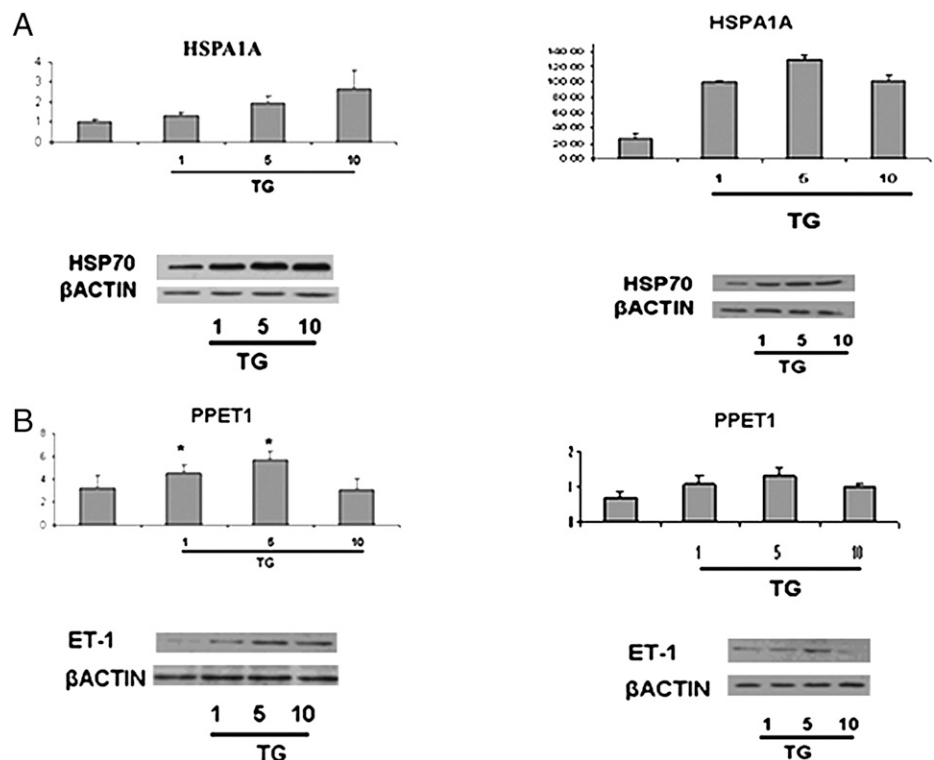
upregulation of HSPs suggested the activation of ER stress and UPR in cells expressing HLA-B35.

Consistent with this possibility, HLA-B35 induced persistent upregulation of BiP at the mRNA and protein levels. BiP, an ER resident protein, is considered to be one of the master regulators of UPR. Misfolded and/or incompletely assembled proteins bind and sequester BiP in the ER, thus shifting the equilibrium away from its

binding to all three UPR transducers inositol requiring kinase 1, protein kinase receptor-like ER kinase, and activating transcription factor 6 (ATF6) (48–52). However, other UPR genes (CHOP, ERO1, and PDI) were not increased. CHOP is a non-ER localized transcription factor and it is induced when the ER stress is extensive or prolonged, leading to apoptosis. Because we did not observe toxicity in cells transduced with Ad-B35 or Ad-B8, absence of ER stress-CHOP pathway and a relatively modest increase of BiP mRNA are consistent with the low level of ER stress in ECs (49–53). PDI facilitates the folding and correct S-S disulfide bond formation of proteins. PDI is regulated by the ERO1, which restores reduced PDI to an oxidized state through disulfide exchange with ERO1 (54). The folding of many proteins depends on the formation of these disulphide bounds; however, we did not find different activation of this pathway in the cells transduced with Ad-B35 when compared with Ad-B8.

The association between ER stress response and expression of the HLA allele was previously observed for HLA-B27, the major risk allele for ankylosing spondylitis (55). Although the mechanism whereby HLA-B27 contributes to development of ankylosing spondylitis is complex and not fully understood, one of the proposed pathogenic events involves protein misfolding. Several studies have shown that HLA-B27 H chain exhibits abnormal properties, including a tendency to misfold and to accumulate in the ER thereby triggering an ER stress response and activation of UPR (56–59). The tendency of HLA-B27 H chain to misfold during the assembly of H chain was further evidenced by the formation of stable complexes with the chaperone BiP (58, 59). Because 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 H chain folding. Interestingly, there is evidence that ER homeostasis is closely related to regulation of inflammatory gene transcription. It was shown that B27/hβ₂ transgenic rat consistently develop colitis and inflammatory peripheral arthritis, as well as frequent inflammatory and fibrotic spinal lesions (60). Furthermore, HLA-B27 misfolding led to generation of

FIGURE 7. Upregulation of ET-1 and HSPA1A in ECV304 (A) and HDMEC (B) cells after TG treatment, total RNA was isolated from ECV304 and HDMEC cells and treated with TG for 24 h (1–5–10 pM). Quantitative RT-PCR was performed with SYBR Green and β-actin as an internal control. The 30 μg total cellular proteins were separated via SDS-PAGE and 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 β-actin using a mAb to β-actin. **p* = 0.05 cells after TG treatment versus untreated cells.



proinflammatory arthritis-causing cytokines and chemokines (55, 61). A link between ER stress and inflammatory response was also reported in other experimental models, including ECs (62). Stimulation of ECs with oxidized phospholipids resulted in activation of UPR and upregulation of IL-8, IL-6, and MCP1 through the ATF4 and x-box binding protein 1-mediated pathway.

Our microarray analysis also revealed significant changes in expression of genes related to IFN signaling pathway, selected proinflammatory genes, including IL-8, IL-6, and several chemokines, as well as cell cycle regulators (Supplemental Table I). However, in contrast to ET-1 and eNOS gene expression, which were consistently altered across all EC lines tested, IFN-related genes could only be validated in selected cell lines (Supplemental Fig. 1), suggesting that additional genetic determinants among the cell lines derived from different individuals may affect specific responses to HLA-B35 in ECs. Because it was shown that the biological consequences of HLA-B27 misfolding may differ considerably depending on the cell types (58, 59), it is possible that more pronounced changes in expression of proinflammatory mediators would occur in immune cells expressing HLA-B35. In fact, we did not observe activation of ER stress and UPR gene response in dermal fibroblast after overexpression of HLA-B35 (or B8) (data not shown). We are planning to examine the effects of HLA-B35 in cells of immune origin in our future studies.

In conclusion, this study shows that the presence of the HLA-B35 allele in ECs results in dysregulated expression of ET-1 and eNOS indicating a pathogenic role of HLA-B35 in PHT. Our data also suggest that upregulation of ET-1 may be directly related to ER stress because TG, a known ER stress inducer, also increased ET-1 at both mRNA and protein levels. However, further studies are needed to elucidate molecular mechanism responsible for the ET-1 upregulation by HLA-B35. ET-1 has been implicated as one of the main pathogenic molecule in the development of SSc-associated PHT and increased levels of ET-1 were found in sera from SSc patients with iPHT. Better knowledge of the pathogenic role of HLA-B35 may help to design preventive therapies in the positive individuals at the early stages of the disease.

Disclosures

The authors have no financial conflicts of interest.

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