Proteasome Inhibitors Enhance Endothelial Thrombomodulin Expression via Induction of Krüppel-Like Transcription Factors

Toyoko Hiroi, Clayton B. Deming, Haige Zhao, Baranda S. Hansen, Elisabeth K. Arkenbout, Thomas J. Myers, Michael A. McDevitt, Jeffrey J. Rade

Objective—Impairment of the thrombomodulin–protein C anticoagulant pathway has been implicated in pathological thrombosis associated with malignancy. Patients who receive proteasome inhibitors as part of their chemotherapeutic regimen appear to be at decreased risk for thromboembolic events. We investigated the effects of proteasome inhibitors on endothelial thrombomodulin expression and function.

Methods and Results—Proteasome inhibitors as a class markedly induced the expression of thrombomodulin and enhanced the protein C activating capacity of endothelial cells. Thrombomodulin upregulation was independent of NF-κB signaling, a principal target of proteasome inhibitors, but was instead a direct consequence of increased expression of the Krüppel-like transcription factors, KLF2 and KLF4. These effects were confirmed in vivo, where systemic administration of a proteasome inhibitor enhanced thrombomodulin expression that was paralleled by changes in the expression of KLF2 and KLF4.

Conclusions—These findings identify a novel mechanism of action of proteasome inhibitors that may help to explain their clinically observed thromboprotective effects. (Arterioscler Thromb Vasc Biol. 2009;29:1587-1593.)

Key Words: proteasome inhibitor • thrombomodulin • Kruppel-like transcription factor • protein C • thrombosis

Patients with hematologic malignancies, especially multiple myeloma, are at high risk for venothromboembolic events (VTE) such as deep venous thrombosis and pulmonary embolus.1 The mechanism responsible for the hypercoagulability associated with myeloma is multifactorial but has been attributed in part to impairment of the thrombomodulin–protein C anticoagulant pathway.2 Thrombomodulin (TM), a membrane glycoprotein abundantly expressed on endothelial cells, binds and alters the active site specificity of thrombin which renders it incapable of enzymatically cleaving fibrinogen or cellular thrombin receptors but enables its activation of circulating protein C.3 Activated protein C (APC), together with its cofactor protein S, proteolytically degrades factors Va and VIIIa of the coagulation cascade, thereby inhibiting further thrombin generation. In myeloma patients, there is evidence for increased release of the TM protein from the endothelial cell membrane into the circulation.3 Loss of TM from the endothelial cell surface, combined with suppressed TM gene expression caused by systemic inflammation, would be expected to impair endothelial APC-generating capacity.5–7

Proteasome inhibitors are a promising new class of agents used for the treatment of multiple myeloma and potentially other types of malignancies.8 The ubiquitin-proteasome system is the major pathway for the nonlysosomal degradation of intracellular proteins and therefore plays a critical role in regulating cellular homeostasis. In a highly regulated series of steps, proteins destined for degradation are covalently modified with ubiquitin, which tags them for recognition by the 26S proteasome complex composed of a 19S regulatory subunit and a 20S proteolytic core.9 The antitumor effect of proteasome inhibitors is thought to be primarily attributable to their ability to inhibit activation of the transcription factor nuclear factor-κB (NF-κB), whose upstream signaling pathways is constitutively active in myeloma cells.10 In quiescent cells, NF-κB is complexed in the cytoplasm to its inhibitor, IκB. After receptor-mediated cytokine stimulation, IκB is phosphorylated, ubiquinated, and then degraded by the proteasome, thereby releasing NF-κB to translocate to the nucleus and transcriptionally activate target genes.11 Proteasome inhibitors effectively block NF-κB activation by inhibiting the proteasomal degradation of IκB.

Emerging data from clinical trials suggest that patients with multiple myeloma who receive proteasome inhibitors as part of their therapeutic regimen have a lower incidence of VTE compared to patients treated with other agents.12 The mechanism underlying this observation is poorly understood. There is evidence that proteasome inhibitors can suppress platelet aggregation, though the effect appears to be independent of inhibition of platelet 20S activity.13 Although proteasome inhibitors have also been shown to stimulate endothelial nitric oxide generation via induction of endothelial nitric oxide synthase (eNOS), the full extent of their effects on endothelial anticoagulant function is largely unknown.14

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We hypothesized that some of the clinically-observed thromboprotective effects of proteasome inhibitors in myeloma patients may be attributable to modulation of the TM–protein C anticoagulant pathway. The goal of the present study was to investigate the effect of proteasome inhibitors on the expression and function of TM in endothelial cells.

Methods

Cell Culture and Reagents

Human umbilical vein endothelial cells (HUVECs; American Type Culture Collection CRL-1730) were maintained in EGM-2 media (Lonza) under 5% CO₂ at 37°C. Cells of passage 2 to 5 were used for all experiments. Bortezomib was provided by Millenium Pharmaceuticals. All other chemicals were purchased from Sigma–Aldrich unless otherwise indicated.

Animal Studies

Animal protocols were approved by the Johns Hopkins Animal Care and Use Committee. C57/HeN male mice weighing 19 to 21 g (Charles River Laboratories, Wilmington, Mass.) were given intraperitoneal injections of bortezomib (0.4 or 0.8 mg/kg) once daily for 7 days. One hour after last injection, mice were anesthetized with isoflurane and organs harvested for mRNA and protein expression analysis.

Real-Time Quantitative PCR

Total RNA was extracted from HUVECs using RNasea Mini kit (Qiagen) or from tissue using TRIZOL Reagent (Invitrogen). After treatment with DNase, samples were subjected to reverse transcription and standard multiplex real-time PCR in duplicate using TaqMan Universal PCR Master Mix Reagents on a 7900HT Sequence Detection System (Applied Biosystems). Levels of mRNA were measured by the standard curve method using pooled total RNA extracted from control cells or tissue. Target mRNA levels were normalized to 18S ribosomal RNA (rRNA). The following primers/labeled probes were purchased from Applied Biosystems: human KLF2 (Hs00360439_g1), mouse KLF2 (Mm00124499_g1), human KLF4 (Hs00358836_m1), mouse KLF4 (Mm00516105_g1), human TM (Hs007097_m1), mouse TM (Mm00437014_s1), and 18S ribosomal RNA control (4308329). Primers/probes for human TM were custom made from the following sequences: forward primer-CCACCAACACCGGGACTGCT; reverse primer-CGTCGATGTCCGTGCAGAT; probe-TGGCCCTGAAGGCTACATCC-TGGACG.

Quantification of NF-κB Activation

NF-κB activation was measured by an ELISA-based method (Trans-AM NF-κB p65; Active Motif) as previously described.5

Western Blot and Immunohistochemical Analysis

Western blotting was performed using primary antibodies to human TM (2375; American Diagnostica), mouse TM (Hs007097; Santa Cruz Biotechnology), and β-actin (#A5441; Sigma–Aldrich) as previously described.5,15 Immunohistochemical staining was performed on formalin-fixed and paraffin-embedded sections of mouse liver as previously described.16 Immunofluorescence staining was performed using primary antibodies to mouse TM (Hs007097; Santa Cruz Biotechnology) and mouse CD31 (#ab28364; Abcam) with fluorescent-labeled secondary antibodies (#A21206; Alexa Fluor 488-labeled anti-Rabbit IgG and #A11058; Alexa Fluor 594-labeled antigoat IgG, Invitrogen).

In Situ Protein C Activation Assay

The capacity to generate activated protein C (APC) in situ was measured on HUVECs as previously described.5

siRNA-Mediated Gene Knockdown

HUVECs (50% to 60% confluent) were transfected with 100 nmol/L of siRNA reagents specific for the human KLF2, KLF4, KLF6, and RelA genes (siGENOME ON-TARGETplus SMARTPool duplex) using DharmaFECT siRNA transfection reagent #1, according to the manufacturer’s instructions (Dharmacon). Control cells were incubated with vehicle (DharmaFECT siRNA transfection reagents) alone or with siGENOME nontargeting siRNA pools. Proteasome inhibitor was added to the medium 48 hours after transfection and incubated for an additional 20 hours.

Nuclear Run-On Assay

A nuclear run-on assay was performed on HUVECs treated with bortezomib or vehicle for 20 hours as previously described.17 mRNA was reverse transcribed then subjected to quantitative PCR analysis as described above.

Statistic Analysis

All data are presented as the mean±SEM. Comparison between 2 groups is by 2-tailed t tests and between multiple groups is by 1 factor ANOVA followed by a Tukey multiple comparison test for intergroup comparisons. Only probability values <0.05, considered to be statistically significant, are shown.

Results

Effect of Proteasome Inhibition on TM Expression and Function

We first characterized the ability of bortezomib,8 a dipeptide boronic acid derivative that is the only proteasome inhibitor approved for human use, to inhibit 20S proteasome activity and NF-κB activation in HUVECs (please see supplemental materials, available online at http://atvb.ahajournals.org). Proteasome and NF-κB activity were suppressed in a dose-dependent manner, with >70% of proteasome inhibition occurring within 3 hours of exposure to bortezomib (supplemental Figure I). In a preliminary experiment, we measured changes in mRNA expression of a series of coagulation-related molecules in HUVECs after a 20-hour exposure to 5 nmol/L bortezomib (supplemental Figure IIA). Compared to controls, bortezomib significantly increased mRNA levels of the anticoagulant molecules TM and eNOS (129±15% and 72±5% of control, respectively, P<0.002 for both), while suppressing levels of the procoagulant molecules protease-activated receptor-1 (PAR-1) and vWF (∼50±1% and −25±2% of controls, respectively, P<0.05 for both) as well as the fibrinolytic molecules urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA; −44±1% and −34±5%, respectively, P<0.01 for both). Bortezomib significantly suppressed cytokine-induced NF-κB activation in a dose-dependent manner, paralleling the degree of proteasome inhibition, and suppressed TNF-α–mediated induction of mRNA encoding E-selectin and vascular adhesion cell molecule-1 (VCAM-1) by >99% and intercellular adhesion molecule-1 (ICAM-1), monocyes chemotactic protein-1 (MCP-1), and tissue factor by >67% (supplemental Figure IIIB).

Based on the above preliminary data, we chose to further explore the effects of proteasome inhibitors on TM expression and function. HUVECs exposed to bortezomib for 20 hours exhibited a dose-dependent increase in TM mRNA and protein expression (Figure 1A and 1B). Similar effects were observed in human aortic endothelial cells (supplemental Figure III). Consequent to increased TM expression we observed a marked rebound that was not diminished by exposure to TNF-α (Figure 1D). TM expression was also induced in a dose-dependent
manner after exposure to the proteasome inhibitors epoxomicin, a natural peptidyl epoxyketone, and MG132, a synthetic tripeptide aldehyde, indicating a class effect (Figure 1E).

To determine whether the NF-κB inhibitory properties of proteasome inhibitors play a role in TM induction, we compared the effects of bortezomib to both chemical and molecular inhibitors of NF-κB activation. Parthenolide, a soluble sesquiterpene lactone NF-κB inhibitor without proteasome inhibitory properties, had no effect on basal TM gene expression whereas bortezomib caused a nearly 4-fold increase despite equipotent NF-κB inhibition (Figure 2A). Both agents effectively prevented TM downregulation by TNF-α. As confirmation, HUVECs were transfected with siRNA targeting the RelA (p65) subunit of NF-κB or a nontarget control. Suppression of RelA gene expression prevented cytokine-induced NF-κB activation but did not diminish TM upregulation by bortezomib (Figure 2B). These data indicate that proteasome inhibitors upregulate TM expression via a mechanism that is independent of NF-κB signaling.

Proteasome Inhibition Induces the Expression of Krüppel-Like Transcription Factors

We next investigated the molecular mechanism by which proteasome inhibitors stimulate TM expression. Pretreatment of HUVECs with cycloheximide abrogated bortezomib-induced TM upregulation, indicating that new protein synthesis is required (Figure 3A). By nuclear run-on assay, exposure to 5 and 10 nmol/L bortezomib increased levels of TM mRNA >10-fold.
(Figure 3B), indicating that proteasome inhibitors induce TM expression via transcriptional activation.

KLF transcription factors, particularly KLF2 and KLF4, have recently been recognized as important mediators of vascular endothelial cell function.18 Both are capable of stimulating the expression of TM and eNOS, similar to the observed effects of proteasome inhibitors.19,20 We therefore investigated whether KLF transcription factors are involved in TM regulation by proteasome inhibitors. HUVECs were first exposed to ascending doses of bortezomib for 20 hours, and changes in the expression of KLF2, KLF4, and KLF6 mRNA measured. Although bortezomib induced all 3 in a dose-dependent manner, the effect was most pronounced for KLF2 and KLF4, with 8- to 18-fold increases at doses 5 nmol/L (Figure 4A). Similar effects on KLF2 and KLF4 mRNA expression were observed in cells treated with MG132 and epoxomicin (supplemental Figure IV). HUVECs were then transfected with siRNA targeting each of the KLF species or a nontarget control before a 20-hour exposure to 5 nmol/L bortezomib. siRNA treatment resulted in ~80% inhibition of target gene expression after bortezomib exposure (supplemental Figure V). Blocking the induction of both the KLF2 and KLF4 genes, but not the KLF6 gene, effectively prevented the upregulation of TM by bortezomib (Figure 4B). Furthermore, combined inhibition of KLF2 and KLF4 appeared to have additive suppressive effects compared to each one alone. These data support the concept that proteasome inhibitor-induced upregulation of TM is mediated by upregulation of the KLF2 and KLF4 transcription factors.

**Systemic Proteasome Inhibition Enhances In Vivo TM Expression**

To investigate whether physiological doses of a proteasome inhibitor could alter TM expression in vivo, mice were administered ascending intraperitoneal doses of bortezomib for 7 days after which TM, KLF2, and KLF4 mRNA levels were measured in several organs (Figure 5A). The most pronounced effect was observed in the liver, where levels of TM mRNA and protein (Figure 5B) increased markedly in a dose-dependent manner (7- and 10-fold over controls at a

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**Figure 2.** Relationship of NF-κB signaling to TM induction. A, HUVECs were pretreated with 8 μmol/L parthenolide (Parth) and 5 nmol/L bortezomib (Bort) for 6 hours before stimulation with ±100 ng/mL TNF-α for 14 hours. The effects on NF-κB activity in whole cell extracts (left) and on TM gene expression (right) are shown (n=3 per group from 1 of 2 duplicate experiments; *P<0.001 vs control + TNF-α; #P<0.001 vs control + vehicle). B, HUVECs were transfected with 100 nmol/L siRNA targeting the RelA gene (RelA) or nontarget control (Non-T) 48 hours before treatment with ±5 nmol/L bortezomib for an additional 20 hours. The effects on RelA gene expression (left), NF-κB activation (middle), and TM gene expression (right) are shown (n=3 per group from 1 of 2 duplicate experiments; *P<0.001 vs Non-T + bortezomib or TNF-α).

**Figure 3.** TM induction by proteasome inhibitors requires new protein synthesis and is attributable to transcriptional activation of the TM promoter. A, Change in TM gene expression in HUVECs pretreated with ±5 μg/mL cycloheximide 1.5 hours before treatment with 5 nmol/L bortezomib for 20 hours at the indicated doses (n=3 per group from 1 of triplicate experiments; *P<0.001 vs 0 nmol/L bortezomib + vehicle). B, Nuclear run-on assay of HUVECs treated with bortezomib at the indicated doses for 20 hours. (n=4 per group; **P<0.005, ***P<0.01 vs 0 nmol/L bortezomib).
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hypertension, and radiation enteropathy.25–27 Inflammatory conditions, including bacterial sepsis, transplant rejection, and tumor necrosis factor (TNF-α) can cause the release of membrane-bound TM from injured endothelial cells, are elevated at the time of diagnosis and fall with treatment.4 TM gene expression is well-recognized to be negatively regulated by inflammatory cytokines, such as TNF-α and interleukin-1β.5 Serum levels of both cytokines are markedly elevated in patients diagnosed with multiple myeloma and, like soluble TM, fall during treatment.6,7 Approximately 10% to 20% of patients with multiple myeloma also develop resistance to the anticoagulant actions of APC which is associated with an increased incidence of VTE.28 In contrast to congenital APC resistance attributable to the presence of the factor V Leiden mutation, the mechanism of acquired APC resistance associated with cancer appears related to increased levels of factors V and VIII which overwhelms the anticoagulant effects of APC.29,30 It is therefore mechanistically plausible that agents such as proteasome inhibitors, with the potential to both increase endothelial TM expression and APC-generating capacity as well as inhibit tissue factor expression, could reduce the risk of VTE in patients with multiple myeloma. Our results provide a conceptual basis for more in-depth in vivo animal and human clinical studies investigating this possibility.

Proteasome inhibition was found to not only stimulate baseline TM expression but also block its downregulation by TNF-α. We previously reported that TM downregulation by cytokines and endotoxin is mediated by activation of NF-κB, which competes for limited cellular quantities of the transcriptional coactivator p300 necessary for TM gene expression.5 As the inhibition of NF-κB is thought to mediate the primary antitumor effects of bortezomib, it was reasonable to speculate that NF-κB signaling might be involved in modulation of TM expression by proteasome inhibitors. The fact that TM upregulation was not diminished by blocking relA expression (Figure 2B) and required new protein synthesis (Figure 3A) ruled out a role for NF-κB signaling in mediating TM upregulation by proteasome inhibitors. Rather, this effect was found to be mediated by induction of the Krüppel-like transcription factors KLF2 and KLF4. KLF2 and KLF4, with the latter exhibiting the greatest induction by systemic proteasome inhibition.

Discussion

The major findings of this study are: (1) proteasome inhibitors as a class markedly stimulate endothelial TM expression resulting in enhanced capacity of endothelial cells to generate APC; (2) proteasome inhibitors effectively prevent the downregulation of TM by inflammatory cytokines; (3) the upregulation of TM by proteasome inhibitors is independent of their NF-κB inhibitory properties but rather mediated by induction of Krüppel-like transcription factors; and (4) systemic administration of a proteasome inhibitor can enhance endothelial expression of KLF2, KLF4, and TM in vivo.

In response to injury or inflammation, the normal anticoagulant properties of the endothelium become impaired and are opposed by the nascent expression of tissue factor which initiates thrombin generation and thrombosis.71 The critical importance of an intact TM–protein C anticoagulant pathway to maintaining endothelial thromboresistance is highlighted by the observation that mice with deletions of TM, protein C, or the endothelial protein C receptor (which facilitates APC generation by the thrombin/thrombomodulin complex) die in utero or in the perinatal period from thrombosis.72–74 The acquired loss of TM expression, with a consequent reduction in APC generating capacity, is thought to be a major contributor to the thrombotic manifestations of several inflammatory conditions, including bacterial sepsis, transplant rejection, and radiation enteropathy.25–27 Dysfunction of the TM–protein C anticoagulant pathway has been implicated in thrombosis associated with multiple myeloma and other malignancies. Levels of soluble TM, caused by the release of membrane-bound TM from injured endothelial cells, are elevated at the time of diagnosis and fall

dose of 0.8 mg/kg/d, respectively, P<0.0001). By immunostaining, TM expression was visibly increased in the liver of bortezomib-treated mice and appeared to localize predominantly to the hepatic sinusoidal endothelial cells (Figure 5C). TM gene expression increased less dramatically in the kidney but was unchanged in the heart and lung. Changes in TM expression were paralleled by changes in the expression of KLF2 and KLF4, with the latter exhibiting the greatest induction by systemic proteasome inhibition.

Figure 4. Induction of TM by proteasome inhibitors is mediated by upregulation of Krüppel-like transcription factors. A, Changes in the levels of KLF2, KLF4, and KLF6 mRNA were measured in HUVECs treated with bortezomib for 20 hours at the indicated doses (n=3 per group of 1 of triplicate experiments; *P<0.01, #P<0.001 vs 0 nmol/L bortezomib). B, Effect of KLF knockdown on TM expression. HUVECs were transfected with 100 nmol/L siRNA targeting the KLF2, KLF4, KLF6 genes and a nontarget (Non-T) control 48 hours before the treatment with ±5 nmol/L bortezomib for 20 hours (n=3 per group of 1 of triplicate experiments).
eNOS expression and blunting of cytokine-induced upregulation of tissue factor, VCAM-1 and E-selectin. It remains possible that the NF-κB inhibitory properties of proteasome inhibitors might still play some role in blocking the downregulation of TM by inflammatory cytokines. However, this effect could also be explained by KLF upregulation, as overexpression of KLF2 also is known to block TM downregulation by inflammatory cytokines. 

Although it has been previously reported that the ubiquitin-proteasome pathway controls the degradation of several of the KLF proteins, the ability of proteasome inhibitors to stimulate KLF gene expression is a novel finding of our study. The mechanism by which this occurs is currently unknown, but the subject of active investigation. The KLFs are a large family of zinc-finger transcription factors that play key roles in regulating a wide array of cellular processes including differentiation, proliferation, apoptosis, and neoplastic transformation. It is possible that some of the antitumor effects of bortezomib may be related to its stimulation of KLF expression rather than solely attributable to its NF-κB inhibitory properties. Consistent with this concept are data indicating that KLF4 and KLF6 can inhibit cellular proliferation through activation of p21WAF1/CIP and may act as a tumor suppressors in gastrointestinal and prostatic tissue. 

An intriguing finding of our study was the observed differential effects of bortezomib on TM induction in various organs. TM induction was most pronounced in the liver and kidney with no significant induction observed in the heart or lung. TM upregulation was only observed in tissue where KLF2 or KLF4 were also upregulated. Susceptibility to thrombus formation in general, and TM expression in particular, is known to vary between different vascular beds. It is therefore possible that there are tissue-specific differences in the molecular pathways by which proteasome inhibitors regulate endothelial thromboresistance. These results, however, may be better explained by the pharmacokinetics of bortezomib. After intravenous injection, the drug is rapidly cleared from the blood with a large volume of distribution. Radiolabeling studies in rats revealed that the liver and kidney have the highest bortezomib uptake, with virtually no uptake in the brain. The maximum tolerated daily intraperitoneal dose (0.8 mg/kg) given to mice in our study was equivalent to approximately half the intravenous bolus dose (1.3 mg/m²) administered to myeloma patients on a biweekly basis in clinical studies. It is possible that alternate dosing schemes utilizing chronic intermittent bolus injections, such as those used in clinical studies, would achieve more widespread and consistent organ effects.
In summary, proteasome inhibitors as a class stimulate the expression of TM and enhance endothelial cell thromboresistance. These effects are mediated via induction of the Kruppel-like transcription factors, KLF2 and KLF4, and may help explain the reduction in thromboembolic events observed in patients who receive proteasome inhibitors for the treatment of plasma cell malignancies.

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Disclosures
None.

References
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SUPPLEMENTAL MATERIAL

Supplemental Methods

Quantification of 20S Proteasome Activity. 20S proteasome activity (kindly performed by Dr. William J. Riordan of Millenium Pharmaceuticals) was measured in whole cell lysates as previously described using a 96-well plate format with a BioTek medel synergy HT microplate reader (BioTek Instruments, Inc Winooski, VT). Proteasome inhibition was determined by calculating the percent change of the chymotryptic specific activity compared to untreated controls.

Quantification of NF-κB Activation. NF-κB activation was measured by an ELISA-based method (Trans-AM NF-κB p65; Active Motif, Carlsbad, CA) as previously described.

Supplemental Figures

Supplemental Figure I. Effect of bortezomib on endothelial cell proteasome and NF-κB signaling activity. A) 20S proteasome activity was measured in the cellular lysates of HUVECs treated with bortezomib at the indicated doses for 20 hours. (n=3 per group, *P <0.01, ** P <0.001 versus 0 nM bortezomib.) B) Time course of 20S proteasome inhibition in the cellular lysates of HUVECs treated with 5 nM bortezomib for up to 24 hours. (n=3 per group, *P <0.001 versus time 0 control.) C) NF-κB activity in whole cell extracts of HUVECs treated with 5 nM bortezomib for 6 hours then stimulated with vehicle or 100 ng/mL TNF-α for 3 hours. (n=3 per group, *P <0.001 versus 0 nM bortezomib + TNF-α.)
Supplemental Figure II. Effects of proteasome inhibition on endothelial expression of coagulation and inflammation-related genes. A) Changes in the expression of coagulation-related genes in HUVECs treated with 5 nM bortezomib for 20 hours. B) Changes in the induction of inflammation-related genes in HUVEC treated with 5 nM bortezomib for 3 hours then stimulation with vehicle or 100 ng/mL TNF-α for an additional 17 hours. Values shown are the mean ± SEM of n=3 samples per group in one of at least two replicated experiments. Target gene expression was normalized to rRNA expression. TM = thrombomodulin; eNOS = endothelial nitric oxide synthase; TFPI = tissue factor pathway inhibitor; HS = heparan sulfate proteoglycan; EPCR = endothelial protein C receptor; tPA = tissue-type plasminogen activator; uPA = urokinase-type plasminogen activator; vWF = vonWillebrand factor; PAR-1 = protease activated receptor-1; PAI-1 = plasminogen activator inhibitor-1; VCAM-1 = vascular cell adhesion molecule-1; ICAM-1 = intercellular adhesion molecule-1; MCP-1 = monocyte chemotactic protein-1; TF = tissue factor.

Supplemental Figure III. Effect of proteasome inhibition on TM expression in human aortic endothelial cells. Dose-related change in TM gene expression, normalized to rRNA, in human aortic endothelial cells treated with bortezomib at the indicated doses for 20 hours. * p<0.001 versus control (0 nM bortezomib). Values shown are the mean ± SEM from three independent experiments.
Supplemental Figure IV. A). Effect of proteasome inhibition on KLF2 and KLF4 expression. Dose-related change in KLF2 and KLF4 gene expression in HUVECs treated with epoxomicin (left panel) and MG132 (right panel) at the indicated doses for 20 hours. *p<0.001 versus control (0 nM proteasome inhibitors).

Supplemental Figure V. Effect of siRNA on KLF expression. HUVECs were transfected with 100 nM siRNAs targeting the KLF2, KLF4, KLF6 genes and a non-target (Non-T) control 48 hours prior to the treatment with ± 5 nM bortezomib for 20 hours. Values shown are the mean ± SEM from three independent experiments. **p<0.01, ***p<0.001 versus control (0 nM proteasome inhibitors, Non-T). †p<0.05, ††p<0.01, †††p<0.001 versus 5 nM bortezomib, Non-T.
Reference List
