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IKK β regulates endothelial thrombomodulin in a Klf2-dependent manner

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Summary

Background—Endothelial thrombomodulin (TM) is critically involved in anticoagulation, anti-inflammation, cytoprotection and normal fetal development. Tumor necrosis factor alpha (TNF α) suppresses TM expression.

Objective—TNF α has been shown to down-regulate TM partly via activation of nuclear factor kappa B (NF- κ B). However, because the TM promoter lacks an NF- κ B binding site, the direct involvement of NF- κ B has been controversial. We investigated the role of the upstream regulatory serine kinase, inhibitory kappa-B kinase- β (IKK β), in TM expression and function with or without TNF α treatment.

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Addendum

R. Pathak and L. Shao analyzed data and wrote the paper. S. Chafekar and W. Feng analyzed and interpreted data. U. Ponnappan designed and performed experiments. L. Fink, D. Zhou and M. Hauer-Jensen conceived the study and revised the paper.

Disclosure of Conflict of Interests

The authors state that they have no conflict of interests.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Effect of cycloheximide (CHX) treatment on BMS-mediated TM overexpression.

Fig. S2. Effect of BMS on TM expression in different primary human endothelial cell lines.

Fig. S3. BMS-mediated TM mRNA stability assay by Actinomycin-D.

Fig. S4. Concentration-dependent TM over-expression after TPCA-1 treatment.

Fig. S5. BMS-mediated TM mRNA expression at different time-points.

Fig. S6. Estimation of TM molecules present on the cell surface membrane of HUVECs and EA.hy926 cells.

Fig. S7. Standard curve of purified human APC.

Fig. S8. Klf2 and Klf4 mRNA expression after knock-down with respective siRNA.

Methods—Inhibition of IKK β was achieved by specific chemical inhibitors, siRNA or shRNA. TM expression was assessed by qRT-PCR, Western blot, flow cytometry, luciferase reporter assay and chromatin immune-precipitation (ChIP) assay. TM function was estimated by generation of activated protein C (APC). NF- κ B activation was determined by immunocytochemistry.

Results and conclusions—IKK β inhibition increased TM expression and function, and attenuated TNF α -mediated TM down-regulation. In contrast, inhibition of downstream canonical NF- κ B protein family members p50 and p65 (RelA) failed to up-regulate TM expression and did not affect IKK β inhibition-mediated TM over-expression. However, knockdown of cRel and RelB, family members of the canonical and non-canonical NF- κ B pathway, respectively, resulted in TM over-expression. IKK β inhibition caused over-expression, increased promoter activity and enhanced binding of Krüppel-like factor 2 (Klf2) to the TM promoter, which positively regulates TM expression. Finally, knockdown of Klf2 completely attenuated IKK β inhibition-mediated TM up-regulation. We conclude that IKK β regulates TM in a Klf2-dependent manner.

Keywords

endothelial cells; inflammation; NF-kappa β ; thrombomodulin; thrombosis

Introduction

Endothelial thrombomodulin (TM) plays a central role in coagulation and inflammation [1,2]. The TM-thrombin complex activates protein C to generate activated protein C (APC) [3]. Although concerns with bleeding and lack of a survival benefit in sepsis have been raised [4], there is some evidence that APC reduces mortality in patients with severe sepsis [5]. TM itself also has intrinsic anti-inflammatory properties, and *in vivo* studies demonstrate beneficial effects of exogenous TM administration [6,7], while clinical trials have shown promising outcomes in patients [8-10]. TM- α appears to be safe and efficacious in patients with DIC [11]. Geiger *et al.* [12] showed that administration of either recombinant TM or APC to mice after exposure to irradiation reduces lethality. Moreover, TM inhibits fibrinolysis by potentiating the activation of thrombin-activable fibrinolysis inhibitor (TAFI), binds High Mobility Group Box 1 (HMGB1) and suppresses complement activation [13,14].

Tumor necrosis factor- α (TNF α) promotes coagulation and inflammation partly by suppressing endothelial TM. Many of TNF α 's inflammatory functions depend on activation of the transcription factor nuclear factor-kappa B (NF- κ B). Although NF- κ B activation has been implicated in TNF α -induced TM repression [15], it is not clear whether TM repression is directly mediated by NF- κ B or by up-stream regulators. Notably, sequence analysis of the TM promoter reveals no potential NF- κ B binding site [15]. Others report that the Krüppel-like transcription factors (Klfs) play a critical role in TM induction after proteasome inhibition [16].

The current study addressed the role of the up-stream regulatory serine kinase of NF- κ B, inhibitory kappa-B kinase- β (IKK β) in TM expression and function. TNF α activates primarily the IKK β -NF- κ B canonical pathway. The IKK β -NF- κ B pathway comprises members of the NF- κ B family, the family of inhibitors of NF- κ B (I κ B), the I κ B kinase

(IKK) complex, and various other regulatory components. The IKK complex is composed of two catalytic subunits, IKK α and IKK β , as well as the regulatory subunit IKK γ [17]. NF- κ B forms a heterodimer/homodimer that resides in the cytoplasm as an inactive complex associated with a member of the I κ B family. Inflammatory stimuli activate the IKK complex. Activated IKK α and/or IKK β phosphorylate I κ B α to cause degradation of I κ B α by the proteasome, thereby allowing NF- κ B to translocate into the nucleus. Inhibition of proteasomal degradation of I κ B α results in TM over-expression via up-regulation of transcription factors Klf2 and Klf4 [16]. However, other factors such as retinoic acid, thrombin, VEGF, heat shock protein and mechanical shear stress can also induce endothelial TM [18].

We hypothesized that IKK β , which regulates canonical NF- κ B family members, may be involved in the regulation of endothelial TM. Our study revealed that inhibition of IKK β indeed induces TM expression and function, and attenuates TNF α -mediated TM down-regulation via modulating Klf2, while knockdown of Klf2 completely blocks IKK β inhibition-dependent TM over-expression.

Methods

Cell lines and reagents

Human embryonic kidney (HEK) 293T cells, EA.hy926 cells and human umbilical vein endothelial cells (HUVECs) were obtained from the American Type Culture Collection (Manassas, VA, USA). Human aortic endothelial cells (HAECs), human microvascular endothelial cell (HMVECs) and human coronary artery endothelial cells (HCAECs) were obtained from Lonza (Walkersville, MD, USA). BMS-345541 (BMS), TPCA-1, Actinomycin-D and cycloheximide were obtained from Sigma-Aldrich (St Louis, MO, USA). CD141 and IgG1, κ Isotype control antibodies, were from BD Biosciences (Sparks, MD, USA). Vectashield mounting medium with DAPI was from Vector Laboratories, Inc. (Burlingame, CA, USA). Human APC was from Anisara (West Chester, OH, USA). To determine cell surface TM antigen a QIFIKIT® kit was obtained from Dako (Carpinteria, CA, USA). RIPA cell lysis buffer, Tris-Glycine SDS gel running buffer, transfer buffer and laemmli SDS 4 $\times$ sample buffer were from Boston BioProducts (Ashland, MA, USA). Recombinant Hirudin was from American Diagnostica Inc. (Stamford, CT, USA). Chromogenix S-2366 was from DiaPharma Group Inc. (West Chester, OH, USA). IKK β , NF κ B1 and goat anti-mouse IgG-HRP conjugated antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Alexa Fluor® 488 goat anti-mouse IgG antibody, TE buffer and DPBS were from Invitrogen (Grand Island, NY, USA). TM, Klf2 and Klf4 antibodies were procured from Novus Biologicals (Littleton, CO, USA). SN-50 was from EMD Biosciences (Billerica, MA, USA). All restriction enzymes were from New England Biolabs Inc. (Ipswich, MA, USA). Human lentiviral vector and pLKO.1 empty vector were from Open Biosystems (Lafayette, CO, USA). Plasmids were from Addgene (Cambridge, MA, USA).

Cell culture and drug treatment

EA.hy926 and 293T cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin solution. HUVEC, HAEC, HMVEC and HCAEC cells were cultured in the EGM-2 Bullet kit (Lonza, Walkersville, MD, USA). Cells were treated with different doses of IKK β inhibitors (BMS and TPCA-1) and/or 20 ng mL⁻¹ of TNF α .

RNA extraction, cDNA preparation, and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using the RNeasy Plus Mini Kit from Qiagen (Valencia, CA, USA). cDNA was prepared using a cDNA Reverse Transcription Kit from Applied Biosystems (Carlsbad, CA, USA). qRT-PCR was carried out using an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Gene expression assays were performed for TM (Hs00264920_s1), RelA (Hs01547741_m1), cRel (Hs00231279_m1), RelB (Hs00232399_m1), Klf2 (Hs00360439_g1), Klf4 (Hs00358836_m1) and 18s (Hs03928990_g1) using the inventoried TaqMan assay from Applied Biosystems with FAM-labeled probes. Expression of each gene of interest was normalized to 18s ribosomal RNA for each of the samples. Relative mRNA expression was calculated using the comparative C_T (2^{-C_t}) method.

Western blot analysis

Cells lysates were electrophoresed on NuPAGE® Novex 4–12% Bis-Tris Gel (Invitrogen, Grand Island, NY, USA) at 100 V of constant current for 2 h after protein quantitation with the BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). Protein samples were transferred onto PVDF membranes (Invitrogen) and the membranes were blocked with 5% non-fat milk from Thermo Scientific. Bound antibody was detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific Pierce).

Lentivirus production, vector construction, and gene knockdown

Lentivirus production, vector construction and gene knockdown were carried out as described elsewhere [19]. According to the sequences of IKK β /WT (wild type) and IKK β /SSEE vectors (Addgene), IKK β /WT and IKK β /SSEE fragments containing PmeI and HpaI sites were generated by PCR using Phusion® Hot Start High-Fidelity DNA Polymerase. PLVUT-GFP- IKK β /WT and PLVUT-GFP- IKK β /SSEE lentiviral vectors were constructed and the resulting vectors were confirmed by DNA sequencing. Control lentiviral pLKO.1 vector and pLKO.1 vectors containing shRNAs for human IKK α (RHS4533-NM_001278) and IKK β (RHS4533-NM_001556) were obtained from Open Biosystems (Huntsville, AL, USA). To establish stable IKK α and IKK β knockdown (KD) EA.hy926 cell lines, EA.hy926 cells were infected twice with the viral particles under centrifugation (900 g) at 35 °C for 30 min. Stably transduced cells were selected with puromycin (2 lg mL⁻¹).

siRNA transfection

Human ON-TARGETplus SMARTpools for Rel-A-, c-Rel-, Klf2 and Klf4-directed siRNA, and a non-specific ON-TARGETplus non-targeting pool for control siRNA were from Dharmacon (Lafayette, CO, USA). EA.hy926 cells were trypsinized, counted, and 5 × 10⁶ cells were electroporated in the presence of the respective siRNA pool at a final

concentration of 25 pmol using the Neon® Transfection System (Invitrogen). Cells were immediately transferred into prewarmed antibiotic-free complete media and incubated for 14–16 h before adding antibiotic-containing media with or without drug. Transfection efficiency was measured after 24–60 h of siRNA treatment by qRT-PCR.

Flow cytometry

Cells were trypsinized, washed with PBS, blocked in PBS with 4% FBS for 30 min, and stained for surface thrombomodulin/CD141 molecules by incubating 100 µL of the cell suspension with phycoerythrin (PE)-labeled primary antibody in PBS with 4% FBS for 30 min. Data were acquired with an LSRII (BD Biosciences) and analyzed with FlowJo Cytometry software from Tree Star Inc. (Ashland, OR, USA). Appropriate isotype controls were used for each experiment. Delta median fluorescence intensity (MFI) was calculated by subtracting the fluorescence of the isotype from that of the stain.

Immunocytochemistry

Cells were grown on four-chamber glass slides. After treatment with 18 µmol L⁻¹ SN-50 and/or 20 ng mL⁻¹ TNFα, cells were fixed with 4% paraformaldehyde in PBS for 30 min, permeabilized with 0.3% Triton-X for 15 min, treated with 1% glycine for 5 min, and blocked overnight with 4% BSA in 1× TBST at 4 °C. The following day, cells were incubated with anti-NF-κB1 (1 : 200) for 4 h at room temperature. After washing with PBS, the specimens were incubated with fluorescent labeled secondary antibody (1 : 5000) for 1 h at 25 °C. Cells were washed five times with 1× TBST and then mounted using Vectashield mounting medium containing DAPI and observed with a fluorescent microscope (Carl Zeiss, Oberkochen, Germany).

Protein C activation assay

Cells (20 000/well of a 96-well plate) were treated with/without BMS for 1 h, followed by 24 h treatment with/without TNFα. Cells were washed with PBS and incubated with thrombin (1 nmol L⁻¹), protein C (0.5 µmol L⁻¹) and reaction buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 2.5 mM CaCl₂ and 0.5% BSA) in a total volume of 60 µL for 1 h. After 1 h incubation at 37 °C, 20 µL hirudin (0.2 unit µL⁻¹) was added to neutralize thrombin. After 10 min, 100 µL chromogenic substrate S-2366 (0.5 mM) was added, and the change in absorbance at 405 nm was measured using a microplate reader (BioTek, Winooski, VT, USA). A standard curve of purified human APC was generated under identical experimental conditions.

Transient transfection reporter assay

Transient transfection was performed using a Neon® Transfection System (Life Technologies, Carlsbad, CA, USA). A total of 1.5–3 µg plasmid DNA was used in transfections, and total DNA was kept constant. TM promoter activity was measured using a Dual Luciferase-reporter assay kit (Promega, Madison, WI, USA), which measures activities of *Firefly* and *Renilla* luciferases sequentially from a single sample. Co-transfected *Renilla* luciferase was used as an internal control. Cells were transfected with PG13-luc (Addgene plasmid 16642) or TM-luc plus *Renilla*-luc. Cells were then harvested, and luciferase

activity was determined using a Dual-Glo luciferase assay system kit (Promega) and normalized to *Renilla* luciferase activity.

Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was performed following the instructions of the Thermo Scientific Pierce Agarose ChIP Kit (Thermo Scientific). Briefly, DNA associated with the specific immunoprecipitate using Klf2 antibody (Protein-Tech) or negative control using normal rabbit IgG was isolated and used as a template for PCR reaction, amplifying a 436-bp region of the TM promoter with the putative Klf2 binding site or the *GAPDH* gene. A 436-bp fragment of the TM promoter between -749 and -313 was amplified by PCR and a 306-bp of the *GAPDH* gene was amplified and used as control. The PCR products were analyzed by 1.5% agarose gel electrophoresis.

Statistical analysis

Results are expressed as means $\pm$ standard deviation (SD). Data were analyzed using Prism software (version 4.0; GraphPad). Statistical comparison of two groups was performed with the unpaired Student's *t*-test or paired Student's *t*-test as appropriate. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test or two-way ANOVA followed by Bonferroni post-tests was used to determine the significance among multiple groups. A probability value of $P < 0.05$ was considered statistically significant.

Results

Pharmacological inhibition of IKK β increases TM expression and attenuates TNF α -mediated TM down-regulation

To assess IKK β 's role in TM expression, IKK β activity was pharmacologically inhibited in EA.hy926 cells using specific inhibitors. IKK β inhibitor BMS increased TM protein expression significantly (Fig. 1A,B). Cycloheximide pretreatment completely blocked BMS-mediated TM over-expression, suggesting that BMS induces *de novo* TM protein synthesis (Figure S1). TPCA-1, another IKK β inhibitor, also significantly increased TM protein expression (Fig. 1C,D). Similar increases in TM expression after BMS treatment were observed at the mRNA level and protein level in four different primary human endothelial cell lines (Figure S2).

Next, we examined whether the effect of IKK β inhibition on TM expression is dose dependent. BMS concentration-dependently increased TM mRNA expression (Fig. 2A). Moreover, BMS increased TM mRNA stability (Figure S3). TM protein levels increased with increasing concentrations of BMS (Fig. 2B) and TPCA-1 (Figure S4).

A time-course study of TM expression in BMS-treated EA.hy926 cells indicated that the increase in TM mRNA expression peaked at 6 h (Fig. 2C and Figure S5). TM protein expression strongly increased at 12 h, as detected by Western blot (Fig. 2D), while flow cytometric analysis revealed that the elevation of surface TM protein reached its highest level at 24 h (Fig. 2E).

TNF α down-regulates TM expression and also activates IKK β . Therefore, we investigated whether pharmacological inhibition of IKK β attenuates TNF α -mediated TM down-regulation. Treatment of EA.hy926 cells with TNF α and BMS significantly reduced and increased TM expression ($P < 0.001$ vs. control), respectively, and importantly, pretreatment with BMS significantly attenuated TNF α -mediated TM down-regulation ($P < 0.001$) (Fig. 3A,B). Like BMS, TPCA-1 also inhibited TNF α -mediated TM suppression (Fig. 3C).

We next used HUVECs to ensure that the results obtained with the immortalized EA.hy926 cell line could be replicated in primary endothelial cells. TNF α -mediated TM reduction was significantly attenuated after BMS pre-treatment (Fig. 3D).

These data clearly indicate that pharmacological inhibition of IKK β inhibits TNF α -dependent TM reduction both in immortalized (EA.hy926) and in primary (HUVEC) endothelial cells. There were significantly fewer TM molecules present on HUVECs (49850 ± 2333.45) compared with EA.hy926 cells (120612.5 ± 1396.54) (Figure S6).

Pharmacological inhibition of IKK β increases TM function

We next investigated whether IKK β inhibition-mediated TM up-regulation also increases the generation of activated protein C (APC). A standard curve of APC was generated using purified human APC (Figure S7). BMS significantly increased APC generation compared with untreated controls ($P = 0.009$) and also significantly ($P = 0.005$) prevented TNF α -mediated inhibition of APC generation in immortal EA.hy926 cells (Fig. 4A) as determined by the paired *t*-test. Similarly, BMS treatment significantly ($P = 0.01$) increased APC generation and significantly ($P = 0.02$) attenuated TNF α -mediated repression of APC generation in primary HUVECs (Fig. 4B). These data confirm that inhibition of IKK β with BMS increases TM function with enhanced APC generation.

Genetic alteration of IKK β modulates TM expression and the effect of TNF α on TM

Although BMS and TPCA-1 are specific inhibitors of IKK β , they may have non-target effects on TM expression. Therefore, the role of IKK β on TM expression was investigated in EA.hy926 cells after IKK β knockdown (IKK β KD), and subsequently in IKK β KD cells reconstituted either with wild-type (WT) IKK β (IKK β KD/WT) or with constitutively active IKK β (IKK β KD/SSEE) plasmids to investigate the effect of restoration or reversal of IKK β deficiency, respectively. The function of IKK α on endothelial TM expression was also studied in IKK α knockdown (IKK α KD) cells. The protein expression of different IKK subunits was confirmed by western blot (Fig. 5A).

Genetic inhibition of IKK β resulted in over-expression of TM at the mRNA and protein levels (Fig. 5B,C), an effect ablated by reconstitution of WT IKK β . Moreover, compared with vector control cells, IKK β knockdown blocked TM down-regulation by TNF α (Fig. 6A). These data clearly indicate that genetic ablation of IKK β , similar to pharmacological inhibition of IKK β , induces endothelial TM expression and inhibits TNF α -mediated TM down-regulation.

We next assessed the effect of constitutively active IKK β (IKK β KD/SSEE) on TM expression. In IKK β KD/SSEE cells, basal TM expression was significantly ($P < 0.0001$)

lower, both at the mRNA and at the protein level, compared with vector-containing control cells (Fig. 5B,D). Unlike IKK β knockdown cells, TNF α treatment significantly reduced ($P < 0.0001$) TM protein expression in IKK β KD/SSEE cells compared with untreated IKK β KD/SSEE cells (Fig. 6B). These results strongly suggest an inverse relationship between IKK β expression level and endothelial TM expression.

We also investigated the role of IKK α in TM expression. Genetic knockdown of IKK α (IKK α KD) in EA.hy926 cells by shRNA caused no change in TM expression, either at the mRNA level or at the protein level (Fig. 5B,E). Unlike the IKK β knockdown, the IKK α knockdown was unable to provide any protection from TNF α -mediated TM reduction compared with vector-transfected control cells (Fig. 6C). Therefore, it is evident that only IKK β plays a major role in modulating endothelial TM expression and in preventing TNF α -mediated TM down-regulation.

Role of inhibition of individual NF- κ B family members on TM expression and on IKK β inhibition-mediated TM up-regulation

Next we investigated the role of different NF- κ B family members involved in the canonical and non-canonical NF- κ B activation pathways in TM expression, and whether IKK β inhibition-mediated TM over-expression is dependent on specific NF- κ B family members. To determine the role of NF- κ B protein family members in TM expression, NF- κ B1/p50 (a canonical NF- κ B family member) nuclear translocation of p50 was inhibited by SN-50. The effect of SN-50 on TM expression, BMS-mediated TM up-regulation and TNF α -mediated TM down-regulation was examined in EA.hy926 cells. TM expression was not increased after SN-50 treatment, whereas BMS treatment increased TM expression in SN-50-treated cells (Fig. 7A). NF- κ B nuclear translocation blockade by SN-50 was also unable to attenuate TNF α -mediated TM down-regulation in EA.hy926 cells as detected by flow cytometry (Fig. 7B), validated by immunocytochemistry (Fig. 7C). These findings indicate that BMS-mediated TM over-expression and TNF α -mediated TM down-regulation are not dependent on p50.

In addition, Rel-A/p65, another important member of the canonical NF- κ B pathway, was knocked down by respective siRNAs in EA.hy926 cells to examine their role on TM expression (Fig. 7D). Knockdown of Rel-A did not induce TM protein expression and BMS treatment for 24 h significantly increased TM protein expression in Rel-A ($P < 0.0001$) knockdown endothelial cells (Fig. 7E). These data clearly indicate that Rel-A of the canonical NF- κ B pathway plays no role in regulating endothelial TM expression. However, shRNA-mediated knockdown of cRel (Fig. 7F), another canonical NF- κ B protein family member, significantly increased TM expression and BMS treatment further enhanced TM expression in cRel knockdown stable cell lines (Fig. 7G).

To determine the role of the non-canonical NF- κ B pathway in TM expression, RelB was knocked down by siRNA (Fig. 7H). RelB knockdown resulted in a significant increase in TM expression and BMS treatment further increased TM expression in RelB knockdown cells (Fig. 7I).

IKK β inhibition resulted in activation of transcription factor Klf2

To determine the potential mechanism of IKK β inhibition-mediated TM over-expression, we next investigated the expression of transcription factors Klf2 and Klf4. Pharmacological and genetic inhibition of IKK β significantly increased Klf2 and Klf4 mRNA expression (Fig. 8A,B). Western blot analysis revealed that BMS treatment increased Klf2 and Klf4 protein expression in EA.hy926 cells in a time-dependent manner (Fig. 8C).

We next determined the effect of Klf2 and Klf4 on TM promoter activity. A 2.2-kb region upstream of the transcription start site (–2.2-kb) of the TM gene was used for promoter-reporter assay. Both Klf2 and Klf4 increased TM promoter activity; however, Klf2 caused stronger TM promoter activity than Klf4 (Fig. 8D). To determine the region within the TM promoter responsible for Klf2- and Klf4-mediated transactivation of TM promoter activity, a series of deletion analyses of the TM promoter were undertaken. As shown in Fig. 8(E), Klf2 and Klf4 are able to transactivate the –2.2-kb-Luc TM promoter. Deletion studies indicate that the majority of activity is retained within the –385-bp-Luc fragment of the TM promoter. However, Klf2- and Klf4-mediated induction of TM promoter activity is essentially lost with the –155-bp-Luc TM promoter construct.

Next we determined which of the two transcription factors play the major role in BMS-mediated TM over-expression in EA.hy926 cells. Knockdown of Klf2 (Figure S8A), but not Klf4 knockdown (Figure S8B), prevented BMS-mediated TM over-expression and double knock-down of Klf2 and Klf4 had a similar effect to that of Klf2 knockdown alone (Fig. 8F), suggesting a critical role of Klf2 in this regard. Lastly, the binding of Klf2 in the TM promoter was verified with the ChIP assay. Klf2 binding in the TM promoter was highly time-dependent and maximum binding occurred at 8 h after 2.5 $\mu\text{mol L}^{-1}$ of BMS treatment (Fig. 8G). Together these findings suggest that Klf2 plays a critical role in IKK β inhibition-mediated TM over-expression.

Discussion

Inhibiting the NF- κ B pathway attenuates TNF α - or LPS-mediated TM down-regulation in various *in vitro* and *in vivo* systems [15,20]. Sohn *et al.* [15] reported that transduction of cells with adenovirus-mediated I κ B α super-repression vector inhibits cytokine-mediated TM down-regulation. Furthermore, blockade of the intrinsic endothelial NF- κ B pathway by conditional over-expression of mutant I κ B α abrogates LPS-mediated TM down-regulation in endotoxemic mice [20]. Curcumin, a natural product that inhibits the upstream regulator of the NF- κ B pathway, inhibits TNF α -mediated TM down-regulation in endothelial cells [21], and proteasome inhibitors that inhibit I κ B α degradation attenuate TNF α -mediated TM repression [16].

Because the 5' un-translated region of the TM promoter lacks an NF- κ B binding site, it was suggested that the NF- κ B pathway may regulate the expression of TM in a manner that does not depend on NF- κ B itself [15]. Our study demonstrates that pharmacological inhibition or genetic down-regulation of IKK β , an upstream regulator of canonical NF- κ B family members, increases endothelial TM expression and functional activity and also attenuates TNF α -induced TM down-regulation. In contrast, inhibition or down-regulation of IKK α , as

well as direct inhibition of some NF- κ B family members has no such effect. Our results are in agreement with those of Hiroi *et al.* [16], who showed that inhibiting I κ B α degradation increased TM expression, while genetic knock-down of the canonical NF- κ B pathway family member Rel-A had no such effect [16]. On the other hand, we did observe that genetic inhibition of cRel and RelB increases TM expression, suggesting a potential role of the NF- κ B pathway. Notably, a previous study reported a sequence within the *TM* gene [15] identical to a non-canonical NF- κ B binding site in the osteocalcin promoter that causes non-canonical NF- κ B-mediated transcriptional repression after TNF α treatment [22].

Analogous to our findings, increasing evidence suggests that IKK β exhibits many biological functions independent of NF- κ B [23]. Moreover, inhibition of upstream regulators of canonical NF- κ B family members induces various transcription factors, notably Klf2 and Klf4, which lead to endothelial TM over-expression without involving canonical NF- κ B family members [16]. We observed that inhibition of IKK β resulted in significant increases in the expression levels of Klf2 and Klf4. Though transient transfection of both Klf2 and Klf4 increased TM promoter activity, BMS-mediated TM induction is found to be mainly dependent on Klf2.

In summary, our results demonstrate that IKK β inhibition up-regulates TM expression, increases APC generation and attenuates TNF α -mediated TM down-regulation in endothelial cells in a Klf2-dependent manner. While knockdown of Rel-A did not up-regulate TM expression and was unable to prevent IKK β inhibition-mediated TM over-expression, cRel and RelB knockdown resulted in TM over-expression, suggesting a probable role of NF- κ B in TM expression. Pharmacological inhibition of IKK β may be a promising approach for treating thrombotic and inflammatory disease conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Pharmacological inhibition of IKK β increases endothelial thrombomodulin (TM) expression. EA.hy926 cells were treated with vehicle (Veh) or 2.5 $\mu\text{mol L}^{-1}$ BMS (A and B) or 5 $\mu\text{mol L}^{-1}$ TPCA-1 (C and D) for 24 h. The expression of TM was quantified by flow cytometry using CD141 immunostaining. Mean fluorescent intensity (MFI) with mean $\pm$ SD of six independent experiments performed in triplicate.

Fig. 2.

BMS dose- and time-dependently increases endothelial thrombomodulin (TM) expression. EA.hy926 cells were treated with different concentrations of BMS for 6 h and TM mRNA level was measured by qRT-PCR, showing dose-dependent increase (A). TM surface protein in EA.hy926 cells was measured by flow cytometry after treatment with various concentrations of BMS for 24 h and was expressed as mean fluorescence intensity (MFI) of CD141 (TM), showing a dose-dependent increase in TM expression up to $5 \mu\text{mol L}^{-1}$ of BMS (B). EA.hy926 cells treated with $2.5 \mu\text{mol L}^{-1}$ BMS for various durations showing that TM mRNA levels reached maximum level at 6 h (C). Western blot analysis in EA.hy926 cells treated with $2.5 \mu\text{mol L}^{-1}$ of BMS for different time intervals revealed that TM protein levels were strongly over-expressed from 12 h onwards (D). Time kinetic study of surface TM expression as detected by flow cytometry analysis of CD141 in EA.hy926 cells treated with $2.5 \mu\text{mol L}^{-1}$ BMS for various time intervals indicates maximum expression at 24 h (E). TM mRNA levels are expressed as fold change over the basal after normalization to 18s rRNA. Mean SD of three independent experiments, six replicates per sample, except the data presented in C, which was the mean of six replicates from two experiments. (a) $P < 0.001$ vs. $0 \mu\text{mol L}^{-1}$ of BMS or 0 h; (b) $P < 0.01$ vs. $5 \mu\text{mol L}^{-1}$ of BMS or 6 h.

Fig. 3.

Pharmacological inhibition of IKK β attenuates TNF α -mediated thrombomodulin (TM) down-regulation in endothelial cells. TM mRNA (A) and TM protein expression (B and C) in EA.hy926 cells pretreated with vehicle, 2.5 μ mol L $^{-1}$ of BMS or 5 μ mol L $^{-1}$ of TPCA-1 for 1 h followed by 24 h treatment with 20 ng mL $^{-1}$ of TNF α or without TNF α (CTL). (D) TM protein expression in human umbilical vein endothelial cells (HUVECs) pretreated with 5 μ mol L $^{-1}$ of BMS for 1 h followed by 24-h treatment with 20 ng mL $^{-1}$ of TNF α or without TNF α (CTL). Data are presented as mean fluorescence intensity (MFI) of CD141.

(a) $P < 0.0001$ vs. vehicle; (b) $P < 0.0001$ vs. $\text{TNF}\alpha$ treated group. TM mRNA levels are expressed as fold change over the basal after normalization to 18s rRNA. Mean SD of three independent experiments consisting of six replicates per sample. $\text{TNF}\alpha$ reduced TM expression, while BMS and TPCA-1 increased TM expression and attenuated $\text{TNF}\alpha$ -induced suppression of TM expression ($P < 0.001$).

Fig. 4.

BMS increases activated protein C (APC) generation and attenuates $\text{TNF}\alpha$ -mediated suppression of APC generation in endothelial cells. APC generation in EA.hy926 cells (A) and human umbilical vein endothelial cells (HUVECs) (B) was measured by chromogenic assay after treating with or without $5 \mu\text{mol L}^{-1}$ of BMS for 1 h followed by 24-h treatment with or without 20 ng mL^{-1} of $\text{TNF}\alpha$. APC generation was determined by turnover of chromogenic substrate over a period of 1 h. Cells treated without thrombin used as control. Mean SD optical density obtained at 405 nm from three independent experiments.

Fig. 5.

Thrombomodulin (TM) expression in genetically modified IKK cell lines. Vector, vector transfected cells; IKKα knockdown (KD), IKKα shRNA transfected cells; IKKβ knockdown (KD), IKKβ shRNA transfected cells; IKKβ KD/WT, IKKβ KD cells reconstituted with wild-type (WT) IKKβ; IKKβ KD/SSEE, IKKβ KD cells reconstituted with a constitutively active form of IKKβ. Representative western blot analysis of IKKα, IKKβ and β-actin expression in EA.hy926 cells with different genetically modified IKK status (A). TM mRNA expression in different genetically modified IKK EA.hy926 cell lines. TM mRNA levels are expressed as fold change over the basal after normalization to 18s rRNA (B). Mean SD of three independent experiments. (a) $P < 0.001$ vs. vector; (b) $P < 0.001$ vs. IKKβ KD. Representative flow cytometric analysis of TM expression in (C) vector and IKKβ KD cells, (D) IKKβ KD/WT and IKKβ KD/SSEE cells and (E) vector and IKKα KD cells.

Fig. 6.

Effects of TNF α on thrombomodulin (TM) expression in different genetically modified IKK cell lines. Expression of TM in vector and IKK β KD cells (A), vector and IKK β KD/SSEE cells (B) and vector and IKK α KD cells (C) analyzed by flow cytometry 24 h after treatment with or without 20 ng mL⁻¹ of TNF α . Mean SD of three independent experiments with six replicates per sample. (a) $P < 0.001$ vs. untreated vector control cells; (b) $P < 0.001$ vs. genetically modified cell lines without TNF α treatment.

Fig. 7.

Effects of pharmacological or molecular inhibition of NF- κ B family members on TM expression and on TNF α -mediated thrombomodulin (TM) down-regulation. TM expression as detected by flow cytometry in EA.hy926 cells pretreated with vehicle or SN-50 (18 $\mu\text{mol L}^{-1}$) for 1 h followed by 24 h treatment with or without BMS (2.5 $\mu\text{mol L}^{-1}$) (A) and with or without TNF α (20 ng mL $^{-1}$) (B), respectively. Representative photomicrographs of NF- κ B1/50 immunofluorescent staining (Green) in EA.hy926 cells without any treatment (I); treated with SN-50 (18 $\mu\text{mol L}^{-1}$) for 24 h (II); treated with 20 ng mL $^{-1}$ of TNF α for 24 h (III); and pretreated with SN-50 for 1 h followed by 24 h treatment with 20 ng mL $^{-1}$ of TNF α (IV), demonstrating that SN-50 treatment inhibited TNF α -stimulated NF- κ B nuclear translocation (C). Rel-A (D) and cRel (F) mRNA expression in EA.hy926 cells after ectopic transfection with siRNA and shRNA knockdown, respectively. Surface TM expression as estimated by flow cytometry after vehicle or 2.5 $\mu\text{mol L}^{-1}$ of BMS treatment for 24 h in Rel-A and cRel knockdown cells (E and G). RelB mRNA expression in EA.hy926 cells after

ectopic transfection with vector control or RelB siRNA (H). Surface TM expression as detected by flow cytometry in vector control or RelB knockdown (KD) cells after treatment with $2.5 \mu\text{mol L}^{-1}$ of BMS for 24 h (I). Rel-A, cRel and RelB mRNA levels are expressed as fold change over the basal after normalization to 18s. Photomicrographs were taken at 639 magnification. (a) $P < 0.001$ vs. vector; (b) $P < 0.001$ vs. RelB KD. Mean SD of three independent assays.

Fig. 8.

Effect of IKK β inhibition on Klf2 and Klf4 expression. EA.hy926 cells treated with 2.5 $\mu\text{mol L}^{-1}$ BMS for 6 h significantly increases Klf2 and Klf4 mRNA expression (A). Relative fold change of Klf2 and Klf4 mRNA in IKK β knockdown (KD) EA.hy926 cells as detected by qRT-PCR (B). Western blot analysis of whole cell lysate of EA.hy926 cells treated with 2.5 $\mu\text{mol L}^{-1}$ BMS for different time intervals reveals time-dependent over-expression of Klf2 and Klf4 protein (C). Klf2 and Klf4 transactivate the thrombomodulin (TM) promoter as detected by transient transfection assay using a 2.2-kb TM-Luc promoter construct and expression plasmids for Klf2 and Klf4 in EA.hy926 cells (D). Normalized (firefly/Renilla) promoter activity is expressed relative to vector transfected control. Sequential deletion analysis of the TM promoter by transient transfection with serially deleted constructs of the TM promoter in EA.hy926 cells reveals that Klf2- and Klf4-mediated TM transactivation requires at least -385-bp of the TM promoter (E). Knockdown of Klf2, but not Klf4, significantly suppresses TM expression in EA.hy926 cells compared with the vector control group, while 2.5 $\mu\text{mol L}^{-1}$ of BMS treatment significantly increases TM expression compared with their respective control groups (F). Double knockdown of

Klf2 and Klf4 had a similar effect to Klf2 knockdown, suggesting Klf4 knockdown has no effect on TM suppression in EA.hy926 cells (F). Chromatin immunoprecipitation (ChIP) assay confirms Klf2 binding to the TM promoter in a time-dependent manner after 2.5 $\mu\text{mol L}^{-1}$ of BMS treatment in EA.hy926 cells; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as control for ChIP assay (G). (a) $P < 0.001$ vs. vector; (b) $P < 0.001$ vs. Klf2 KD or Klf4 KD or Klf2 + Klf4 KD. Mean $\pm$ SD of at least three independent experiments.