Antithrombotic Effects of Nur77 and Nor1 Are Mediated Through Upregulating Thrombomodulin Expression in Endothelial Cells

Ping Yang,* Xin Wei,* Jian Zhang, Bing Yi, Guan-Xin Zhang, Litian Yin, Xiao-Feng Yang, Jianxin Sun

Objective—Thrombomodulin is highly expressed on the luminal surface of vascular endothelial cells (ECs) and possesses potent anticoagulant, antifibrinolytic, and anti-inflammatory activities in the vessel wall. However, the regulation of thrombomodulin expression in ECs remains largely unknown.

Approaches and Results—In this study, we characterized nuclear receptor 4A family as a novel regulator of thrombomodulin expression in vascular ECs. We demonstrated that both nuclear receptors 4A, Nur77 and Nor1, robustly increase thrombomodulin mRNA and protein levels in human vascular ECs and in mouse liver tissues after adenovirus-mediated transduction of Nur77 and Nor1 cDNAs. Moreover, Nur77 deficiency and knockdown of Nur77 and Nor1 expression markedly attenuated the basal and vascular endothelial growth factor–stimulated thrombomodulin expression. Mechanistically, we found that Nur77 and Nor1 increase thrombomodulin expression by acting through 2 different mechanisms. We showed that Nur77 barely affects thrombomodulin promoter activity, but significantly increases thrombomodulin mRNA stability, whereas Nor1 enhances thrombomodulin expression mainly through induction of Kruppel-like factors 2 and 4 in vascular ECs. Furthermore, we demonstrated that both Nur77 and Nor1 significantly increase protein C activity and inhibit tumor necrosis factor α–induced prothrombotic effects in human ECs. Deficiency of Nur77 increases susceptibility to arterial thrombosis, whereas enhanced expression of Nur77 and Nor1 protects mice from arterial thrombus formation.

Conclusions—Our results identified nuclear receptors 4A as novel regulators of thrombomodulin expression and function in vascular ECs and provided a proof-of-concept demonstration that targeted increasing expression of Nur77 and Nor1 in the vascular endothelium might represent a novel therapeutic approach for the treatment of thrombotic disorders. (Arterioscler Thromb Vasc Biol. 2016;36:361-369. DOI: 10.1161/ATVBAHA.115.306891.)

Key Words: anticoagulants ■ endothelial cells ■ thrombomodulin ■ thrombosis ■ tumor necrosis factor

The vascular endothelium plays pivotal roles in maintaining the health and homeostatic states of the mammalian circulatory system via the complementary actions of endothelial cell (EC)–derived vasoactive factors.1 Dysregulation of these vasoactive factors has serious clinical outcomes ranging from endothelial dysfunction to atherosclerosis, stroke, and thrombosis.2 One of these factors is namely thrombomodulin, which is highly expressed on the surface of all vascular ECs and mediates the anticoagulant and anti-inflammatory pathways in vascular endothelium.3 For instance, thrombomodulin has been shown to bind to thrombin to potentiate the protein C activation cascade to generate activated protein C (APC), which in turn exerts potent anticoagulant effects through the proteolytic inactivation of factors Va and VIIIa.4 In addition, through binding to thrombin, thrombomodulin has been shown to inhibit inflammation through reducing circulating levels of thrombin as well as switching its substrate specificity toward of thrombin activatable fibrinolysis inhibitor to yield activated thrombin activatable fibrinolysis inhibitor, hence exerting anti-inflammatory and antifibrinolytic activities.5–7 Recently, much attention has been devoted to the therapeutic values of recombinant thrombomodulin in various human diseases. Experimental studies demonstrate that recombinant soluble thrombomodulin is effective in attenuating neointimal hyperplasia after balloon injury and inflammatory disorders, such as heat stroke and radiation toxicity.5,9 Moreover, recent clinical trials also demonstrate the potential therapeutic benefits of thrombomodulin in the treatment of DIC (disseminated intravascular coagulation)10,11 and

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transplantation-associated sepsis. Therefore, understanding the molecular mechanisms underlying the regulation of thrombomodulin expression and function in vasculature is of great scientific and therapeutic interests.

The nuclear orphan nuclear receptor 4A (NR4A) subfamily includes 3 members, namely Nur77 (also called NR4A1), Nur1 (NR4A2), and Nor1 (NR4A3). These receptors have no known ligands and, therefore, are sometimes referred to as orphan nuclear receptors. As immediate-response genes, the NR4A receptors play essential roles in a variety of critical biological processes, including proliferation, differentiation, cell survival, inflammation, glucose and lipid metabolism, and vascular remodeling. Recently, much attention has been paid to the functional role of NR4A receptor in cardiovascular system. In vascular ECs, expression of NR4A receptors, Nur77 and Nor1, is increased by hypoxia, tumor necrosis factor α (TNF-α), and vascular endothelial growth factor (VEGF) to modulate EC growth, angiogenesis, and inflammation. In vascular smooth muscle cells, expression of NR4A receptors, Nur77 and Nor1, is regulated by multiple mitogens, including PDGF (platelet-derived growth factor), serum, and oxidized low-density lipoprotein, thus implicating the functional significance of these receptors in proliferative vascular disorders. In contrast to the functional role of Nur77 and Nor1 in vascular biology, Nur1 has been shown to play critical roles in the survival of the dopamine neurons and development of schizophrenia.

Although NR4A receptors are critical in EC proliferation, inflammation, and angiogenesis, their importance in regulating endothelial thrombotic functions has yet to be explored. In this study, we identified both NR4A receptors, Nur77 and Nor1, as potent positive regulators of thrombomodulin expression in vascular ECs and demonstrated that overexpression of NR4A receptors substantially attenuates TNF-α-induced downregulation of thrombomodulin expression in vitro and inhibits arterial thrombus formation in vivo.

Materials and Methods
Materials and Methods are available in the online-only Data Supplement.

Results
Thrombomodulin Expression Is Increased by Nur77 and Nor1
Because both NR4A receptors, Nur77 and Nor1, have been shown to play important roles in regulating endothelial functions, such as angiogenesis, inflammation, and vascular barrier function, we hypothesized that these receptors may regulate endothelial function through affecting the expression of thrombomodulin, which is a key regulator implicated in EC homeostasis. To test this hypothesis, human umbilical vein endothelial cells (HUVECs) were transduced with either Ad-Nur77 or Ad-Nor1 at indicated multiplicity of infection; 48 hours after adenovirus transduction, the expression of thrombomodulin was determined by both quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot. As shown in Figure 1A–1D, in a dose-dependent manner, transduction of Ad-Nur77 or Ad-Nor1 substantially increased thrombomodulin expression at both mRNA and protein levels. Furthermore, we found that Nur77 overexpression barely affected the Nor1 expression, and vice-versa (data not shown). Together, these data suggest that NR4A receptors, Nur77 and Nor1, are potent regulators of thrombomodulin expression in vascular ECs.

NR4A Receptors, Nur77 and Nor1, Increase Thrombomodulin Expression Through Distinct Mechanisms
NR4A receptors have been shown to function as transcriptional factors or co-factors to regulate gene expression. To determine the molecular mechanism by which Nur77 and Nor1 induce thrombomodulin expression, we first attempted to determine whether Nur77 and Nor1 affect thrombomodulin expression at transcriptional levels. To this end, we constructed thrombomodulin promoter luciferase reporter gene. As shown in Figure 2A and 2B, Nur77 overexpression barely affected thrombomodulin promoter activity, but significantly prolonged the thrombomodulin mRNA half-life (from 2.9±0.6 hours to 5.1±0.8 hours, P<0.05, n=3), as determined by qRT-PCR in HUVECs. Similar results were obtained in mouse lung microvesSEL ECs (Figure I in the online-only Data Supplement). To further corroborate the thrombomodulin promoter-driven luciferase assays, we transduced ECs with adenovirus bearing dominant-negative mutant (DN-Nur77), which lacks the transcriptional activation domain of Nur77.

As shown in Figure 2C, overexpression of DN-Nur77 also markedly increased thrombomodulin expression to the extent similar to wild-type (WT) Nur77 in HUVECs, further indicating that Nur77 regulates thrombomodulin expression occurred at the post-transcriptional levels. In contrast to Nur77, overexpression of Nor1 significantly decreased thrombomodulin promoter activity (Figure 3A), but barely affected thrombomodulin mRNA half-life (Figure 3B). Computer-based sequential analysis of thrombomodulin promoter (Figure II in the online-only Data Supplement) did not reveal any conservative Nor1-binding sites located in the human thrombomodulin promoter region, suggesting that Nor1 may regulate thrombomodulin promoter activity indirectly through action on other transcriptional factors. Because Kruppel-like factors (KLFs) are critically involved in thrombomodulin expression at transcriptional levels, we attempted to determine whether Nor1 affects expression of KLFs in ECs. As shown in Figure 3C, in a dose-dependent manner, overexpression of Nor1 significantly increased KLF2 and KLF4, but not KLF6 expression in HUVECs, as determined by qRT-PCR.
Furthermore, small interference RNA–mediated knockdown of KLF2, KLF4, or both markedly attenuated Nor1-induced thrombomodulin expression in ECs (Figure 3D), further suggesting that Nor1 increases thrombomodulin expression through inducing KLF2 and KLF4 transcriptional activities in vascular ECs.
NR4A Receptors, Nur77 and Nor1, Are Involved in VEGF-Induced Thrombomodulin Expression in ECs

VEGF has been shown to upregulate the expression of both thrombomodulin and NR4A receptors in vascular ECs, which prompted us to investigate whether upregulation of NR4A receptors is attributed to the VEGF-induced thrombomodulin expression in ECs. Consistent with previous studies, we demonstrated that in a time and dose-dependent manner, VEGF165 significantly increased thrombomodulin expression in HUVECs (Figure A and B). To investigate the functional role of NR4A receptors in endothelial thrombomodulin expression, we performed a loss-of-function study by using a small interference RNA approach. Transfection of Nur77- or Nor1-specific small interference RNA markedly inhibited both basal and VEGF165–induced expression of Nur77 or Nor1 in HUVECs, as determined by both qRT-PCR (Figure III in the online-only Data Supplement) and Western blot (Figure 4C). Accordingly, both basal and VEGF165–induced thrombomodulin expression was significantly attenuated in Nur77 or Nor1 knockdown cells (Figure 4C and 4D), suggesting that both Nur77 and Nor1 are essentially involved in the basal and VEGF165–induced thrombomodulin expression in ECs.

To further substantiate the functional significance of NR4A receptor in thrombomodulin expression, we examined the expression of thrombomodulin in Nur77 knockout mice. As shown in Figure 4E, thrombomodulin expression was significantly decreased in the heart, liver, lung, and aorta of Nur77 knockout mice, as determined by qRT-PCR. In an ex vivo experiment, VEGF165 stimulation significantly increased thrombomodulin expression in the aorta of the WT mice, but not in the aorta of Nur77 knockout mice (Figure 4F). Together, these results suggest that Nur77 is critically involved in the expression of thrombomodulin in the vascular wall.

Overexpression of Nur77 and Nor1 Promotes Protein C Activity and Inhibits Blood Clotting In Vitro

Thrombomodulin is a key regulator of the thrombin-mediated activation of the natural anticoagulant APC. To investigate whether NR4A receptors, Nur77 and Nor1, affect the function of thrombomodulin in ECs, we determined the enzymatic activity by measuring the production of APC. As shown in Figure 5A, transduction of Ad-Nur77 or Ad-Nor1 significantly increased the activity of APC in HUVECs. Consistent with previous reports, we found that TNF-α treatment significantly decreased thrombomodulin expression in HUVECs, which was substantially reversed in ECs overexpressing either Nur77 or Nor1 (Figure 5B). Interestingly, overexpression of Nur77 and Nor1 markedly inhibited the expression of tissue...
factor under both basal and TNF-α–stimulated conditions (Figure IV in the online-only Data Supplement). To further determine the functional consequence of Nur77 and Nor1–induced thrombomodulin expression in ECs, we performed an in vitro blood clotting assays. HUVECs were transfected with Ad-LacZ, Ad-Nur77, or Ad-Nor1 for 48 hours, and then the clotting time of recalcified human plasma was determined in the presence or the absence of TNF-α stimulation. As shown in Figure 5C, exposure of recalcified human plasma to HUVECs transduced with Ad-Nur77 or Ad-Nor1 significantly prolonged the clotting time, when compared with that in LacZ–infected cells. Moreover, TNF-α stimulation significantly shortened the clotting time, and this prothrombotic effect was markedly prevented in HUVECs overexpressing either Nur77 or Nor1. Together, these results suggest that NR4A receptors, Nur77 and Nor1, are critically involved in modulating thrombomodulin function in vascular ECs under both baseline and inflammatory conditions.

Deficiency of Nur77 Enhances Susceptibility to Arterial Thrombosis

We next examined the effect of Nur77 deficiency on susceptibility to arterial thrombosis by using the Rose Bengal laser injury method.27 Baseline white blood cell, hemoglobin, and platelet counts were similar in Nur77 knockout mice and in their WT littermates (Figure 6A). After photochemical injury of the carotid artery, the time to occlusive thrombus formation was significantly faster in Nur77 knockout mice than in WT mice (22.3±4.9 versus 34.0±5.8 minutes, respectively; \( P < 0.05 \); Figure 6B). Tail bleeding time was similar in Nur77 knockout mice and in their WT littermates (Figure 6C). To further determine whether Nur77 and Nor1 could be potential
therapeutic targets for thrombotic orders, the mice were transduced with either Ad-Nur77 or Ad-Nor1 via a tail vein injection. The expression of thrombomodulin in the liver was determined at 6 days after virus injection. Similar to the in vitro studies, the in vivo transduction of either Ad-Nur77 or Ad-Nor1 markedly increased the expression of thrombomodulin in mouse liver tissues, as determined by Western blot (Figure 6D). Importantly, mice transduced with Ad-Nur77 and Ad-Nor1 displayed a significantly prolonged time to occlusive arterial thrombus formation when compared with Ad-LacZ group (43.2±6.5, 45.3±7.2 versus 32.8±6.1 minutes, respectively; P<0.05; Figure 6E). Together, these data demonstrate that Nur77 deficiency increases susceptibility to arterial thrombosis, whereas targeted overexpression of Nur77 and Nor1 in vivo prevents thrombus formation.

Discussion

NR4A receptors are immediate-early genes that are regulated by various physiological stimuli, including growth factors, hormones, and inflammatory signals in cardiovascular system.17 An increasing number of studies have demonstrated that NR4A receptors play important roles in the development of various cardiovascular diseases, including atherosclerosis, restenosis, angiogenesis, and heart failure.15,21,28–30 Previously, our studies identified Nur77 as a potent inhibitor of vascular inflammation through inhibiting nuclear factor-κB (NF-κB) transcriptional pathway.16 Here, we provide further evidence highlighting the critical importance of NR4A receptors, Nur77 and Nor1, in regulating thrombomodulin expression in vascular ECs as well as arterial thrombus formation in vivo.

Thrombomodulin is one of the important anticoagulant substances produced by the vascular endothelium to maintain blood fluidity. The binding of thrombomodulin to thrombin has shown to potentiate the generation of activate protein C and facilitate the formation of activated thrombin activatable fibrinolysis inhibitor, thus exerting potent anticoagulant, anti-inflammatory, and antifibrinolytic benefits.4,31 Indeed, homozygous mice with thrombomodulin mutant (Glu404Pro) exhibit reduced ability to generate APC, hence resulting in severe hypercoagulable state and massive thrombosis.32 Despite its importance in the regulation of vascular homeostasis, the molecular mechanisms regulating thrombomodulin expression in vasculature remain largely obscured. In this study, our data provide compelling evidence implicating the functional significance of NR4A receptors in regulating thrombomodulin expression in vascular ECs and thrombotic function in vivo. Importantly, we found that overexpression of Nur77 and Nor1 robustly increase the thrombomodulin expression, attenuates the TNF-α–induced prothrombotic states in ECs, and inhibits arterial thrombus formation in vivo. Furthermore, we demonstrated that Nur77 deficiency significantly increases susceptibility to arterial thrombus formation, further implicating importance of NR4A receptors in maintaining vascular homeostasis in endothelium.

Several molecular mechanisms at transcriptional, post-transcriptional, and post-translational levels have been implicated in the regulation of thrombomodulin expression and function in the vascular endothelium. At transcriptional levels, hypoxia,13 oxidized low-density lipoprotein,34 phorbol esters (PMA),35 cyclic adenosine monophosphate,35 and TNF-α26 have been shown to downregulate endothelial thrombomodulin expression, whereas statins,36 VEGF,25 retinoic acid,37 and heat shock38 have been shown to upregulate thrombomodulin expression. Particularly, KLFs including KLF2 and KLF4 have been shown to play essential roles in the regulation of thrombomodulin expression at transcriptional levels.22,24,39 For example, laminar shear stress, statins, proteasome, and inhibitors have been shown to enhance endothelial thrombomodulin expression via induction of KLF transcriptional activities.22,24,39 Indeed, KLF2 and KLF4 have multiple endothelial protective effects, which may collectively contribute to the antithrombotic effects of Nor1 in ECs. Nevertheless, identification of Nor1 as a novel regulator for KLF2 and KLF4 expression in vascular ECs is of great importance. Although the thrombomodulin
promoter does not contain a classic NF-κB consensus motif, the activation of NF-κB has been implicated in the cytokine-induced repression of thrombomodulin expression in ECs, possibly through competing p300 for its binding to the thrombomodulin promoter.26 Furthermore, miRNAs, such as miR-92a, have been recently reported to regulate thrombomodulin expression through post-transcriptional mechanisms.40 In this study, we demonstrate that NR4A receptors, Nur77 and Nor1, are critically involved in the regulation of basal and VEGF-stimulated thrombomodulin expression in ECs. Although the members of the NR4A subgroup are well conserved in the DNA-binding domain (≈91–95%), these receptors normally exert different or even opposite biological effects through interacting with other co-factors.17 Interestingly, in this study, we found that Nur77 and Nor1 regulate thrombomodulin expression through distinct molecular mechanisms. Nur77 increases thrombomodulin expression predominantly through increasing thrombomodulin mRNA stability, whereas Nor1 involves an induction of KLF2 and KLF4 in ECs. Indeed, depending on the nature of the stimuli and their cellular localization, NR4A receptors can exert biological effects through both genomic and nongenomic actions.21 For instance, in the nucleus, NR4A receptors can function as transcription factors to regulate the expression of the target genes by binding to NGFI-B (nerve growth factor-induced clone B) response element (5′-AAAAAGGTCA-3′) or Nur-responsive element (5′-TGATATTTTX6AAAGTCCA-3′).14 In this study, our data suggest that such a genomic action is not likely involved in the stimulatory effects of Nur77 on thrombomodulin expression because DN-Nur77, which lacks the functional N-terminal AF-1 domain of Nur77, induces thrombomodulin expression to a similar extent as did WT Nur77. In addition, the sequential analysis of the thrombomodulin promoter did not reveal any NR4A receptor-binding consensus sites, further suggesting that the nongenomic action of Nur77 may contribute to its stimulatory effect on thrombomodulin expression. In contrast to Nur77, Nor1 augments the thrombomodulin promoter activity indirectly through induction of both KLF2 and KLF4 in ECs. Future studies will be aimed at elucidating the molecular mechanisms underlying increasing thrombomodulin mRNA stability by Nur77 and induction of KLFs by Nor1 in vascular ECs.

Figure 6. Nur77 gene deficiency increases susceptibility to thrombus formation in vivo. A, white blood cell (WBC) count, hemoglobin (HB), and platelet (PLT) counts in Nur77 knockout (KO) mice and their wild-type (WT) littermates (n=7 per group). B, Nur77 gene deficiency (Nur77 KO) enhances susceptibility of the mice to carotid artery thrombus formation (n=7–9 per group). *P<0.05 vs WT mice. C, Tail bleeding time was not changed in Nur77 KO mice and their WT littermates (n=7 per group). D, Mice were administrated with 100-μL Nur77 and Nor1 adenoviruses (Ad-Nur77 and Ad-Nor1, 1×10¹¹ pfu/mL) by a tail vein injection; 6 days after virus transduction, mice were sacrificed and liver tissues were collected. Thrombomodulin (TM) protein expression in mouse liver tissues was determined by Western blot analysis (n=8 per group). *P<0.05 vs Ad-LacZ. E, Mice were administrated with either 100 μL Ad-LacZ or Ad-Nur77 or Ad-Nor1 (1×10¹¹ pfu/mL) by a tail vein injection; 6 days after adenovirus transduction, carotid artery thrombosis was performed (n=7–9 per group). *P<0.05 vs Ad-LacZ group. NS indicates not significant.
overexpression of Nor1 barely affected TNF-α-stimulated NF-kB activation as determined by EMSA (the gel electrophoresis mobility shift assay; Figure IV in the online-only Data Supplement). On the basis of these observations, it is attempted to speculate that in unstimulated ECs, Nur77 increases thrombomodulin expression mainly through increasing thrombomodulin mRNA stability, whereas in TNF-α-stimulated cells, Nur77 increases thrombomodulin expression most likely through inhibiting NF-kB activation or through both inhibiting NF-kB activation and increasing thrombomodulin mRNA stability. The molecular mechanism underlying induction of thrombomodulin expression by Nor1, however, is different, and it may involve the upregulation of the KLF2 and KLF4 transcriptional activities under both basal and TNF-α-stimulated conditions, which merits further investigation. Furthermore, VEGF has been shown to potently increase basal thrombomodulin expression and block IL-1β-induced suppression of thrombomodulin expression in ECs, which is believed to be essential for maintaining local hemostasis during angiogenesis and inflammatory processes.29 The molecular mechanism underlying VEGF-induced thrombomodulin expression, however, has never been explored thus far. Because VEGF is a potent stimulus for the expression of NR4A receptors in ECs,15 we attempted to speculate that NR4A receptors may also mediate VEGF-induced thrombomodulin expression in vascular ECs. Indeed, we show that both basal and VEGF-induced thrombomodulin expressions were substantially attenuated in Nur77 and Nor1 knockdown cells as well as in the aorta of Nur77−/−deficient mice. Collectively, these data clearly demonstrate that NR4A receptors, Nur77 and Nor1, are essential regulators of endothelial thrombomodulin expression under various pathophysiological circumstances.

In conclusion, we identified NR4A receptors, Nur77 and Nor1, as critical regulators of endothelial thrombomodulin expression and arterial thrombosis in vivo and further implicate the essential roles of NR4A receptors in the pathological processes of the inflammatory and thrombotic diseases. Collectively, our results provide a proof-of-concept demonstration that targeting NR4A receptors, Nur77 and Nor1, in the vascular wall might have a therapeutic role in ameliorating the onset or progression of thrombotic disorders.

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Disclosures

None.

References


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Supplemental Materials and Methods

**Cell Culture**— Human umbilical vein endothelial cells (HUVECs) (ATCC, Manassas, VA, USA) were cultured in endothelial basal medium-2 (EBM-2) with endothelium cell growth factor cocktail (ATCC, Manassas, VA, USA), which contains Bovine Brain Extract (BBE), rhEGF, L-glutamine, Heparin sulfate, Hydrocortisone hemisuccinate, 2% Fetal Bovine Serum, and Ascorbic acid (ATCC, PCS100040). EA.hy926 cells were purchased from ATCC and maintained in Dulbecco Modified Eagle Medium (DMEM) with 10% FBS (Invitrogen, Grand Island, NY, USA), 1% penicillin/streptomycin (Invitrogen, Grand Island, NY, USA).

**Animal studies**— C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). This study was reviewed and approved by the Institutional Animal Care and Use Committee at Thomas Jefferson University. 100 μl Ad-LacZ, Ad-Nur77 or Ad-Nor1 (10¹¹ pfu/ml) was injected into 10-12 weeks old male mice by tail vein injection. 6 days after virus injection, mice were anesthetized with isoflurane and the liver was harvested for the detection of TM expression by western blot analysis.

**TM promoter plasmid constructs**—To generate TM promoter reporter plasmid, human TM promoter fragment was amplified by PCR using the following primers (forward primer: 5'-GCGCAGATCTGGCTCTTCAGTGCCCTTT-3', reverse primer: 5'-GCGCAAGCTTCTCTCCTGTCCGTCCCA-3') from human genomic DNA (Clontech, Mountain View, CA, USA) and then cloned into the luciferase reporter plasmid pGL3-Basic (Promega, Madison, WI, USA).

**Adenovirus Construction**—Adenoviruses harboring wild-type Flag-tagged Nur77 (Ad-Nur77), dominant –negative Nur77 (DN-Nur77) and Nor1 (Ad-Nor1) were made using AdMax (Microbix, Mississauga, Ontario, Canada) as described previously 1. The viruses propagated in Ad293 cells and purified using CsCl2 banding followed by dialysis against 20 mM Tris-buffered saline with 10% glycerol. Titering was performed on Ad293 cells using Adeno-X Rapid Titer kit (Clontech, Mountain View, CA, USA) according to the manufacturer’s instruction.

**Western Blot Analysis**—Cellular protein were extracted in RIPA buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS) supplement with proteinase inhibitor mixture containing 2 mM phenylmethylsulfonyl fluoride (PMSF), 20 μg/ml aprotinin, 10 μg/ml leupeptin. Cell lysates were rocking 1 h at 4°C, then centrifuge at 14,000 rpm at 4°C for 20 min. Cell lysates were then subjected to SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA,USA). Blots were blocked with 5% nonfat milk in PBS and then incubate with diluted anti-TM antibody (1:200, 1 μg/ml, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Nur77 antibody (1:500, 0.4 μg/ml, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Nor1 antibody (1:500, 0.4 μg/ml, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Flag antibody (1:1000, 0.5 μg/ml, Genescript, Piscataway, NJ, USA), anti-GAPDH antibody (1:1000, 0.2 μg/ml, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Blots were visualized on an Odyssey Imaging System (Li-Cor Biosciences,
Lincoln, NE, USA). The intensity of the bands was quantified by the Odyssey software (Li-Cor Biosciences, Lincoln, NE, USA).

**Transient Transfection and Luciferase Assay**— EA.hy926 cells seeded in 6- or 24-well plates were transfected with Ad-Nur77 or Ad-Nor1 virus for 24 hr. The TM promoter reporter plasmid was then transfected using Lipofectamine™ LTX (Invitrogen, Grand Island, NY, USA) transfection reagent. 48 hours after transfection, cells extract were prepared with lysis buffer (25mM Tris-phosphate (pH 7.8), 2mM DTT, 2mM 1,2-diaminocyclohexane-N,N,N′,N′-tetraacetic acid, 10% glycerol, 1% Triton X-100) (Promega, Madison, WI, USA), and the luciferase activity was determined by luminescence counter (PerkinElmer Life Sciences, Waltham, MA, USA) using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the instructions of the manufacturer. Firefly luciferase activity was normalized for transfection efficiency by corresponding Renilla luciferase activity. All transfection experiments were performed at least five times in duplicate.

**Transfection of Small Interfering RNA (siRNA)**— Human Nur77 (Sigma SASI-Hs02-00333289), human Nor1 siRNA (Sigma SASI-Hs01-00091655), and negative control siRNA (Mission siRNA Universal Negative Control) (Sigma-Aldrich, St. Louis, MO, USA) were transfected into HUVECs with Lipofectamine RNAiMAX transfection Reagent (Invitrogen, Grand Island, NY, USA) in OPTI-MEM (Thermo Fisher Scientific, Grand Island, NY, USA) according to the manufacturer’s recommendation. Four hours later, the medium was replaced by EBM-2 and cultured for an additional 48 hr and then treated with or without 100 ng/ml VEGF165 (PeproTech, Rocky Hill, NJ) for 3 hr or 24 hr as indicated, and cells were harvested for protein or RNA extraction to assess the expression of target genes.

**Quantitative Real Time PCR (qRT-PCR)**—Total RNA was extracted from HUVECs or mouse tissues using TRIZOL reagent kit (Invitrogen). qRT-PCR analysis was performed as described previously. Briefly, cDNA was synthesized from total RNA using High Capacity cDNA Archive Kit (Applied Biosystem). qRT-PCR was performed using MyiQTM Single-Color Real-Time PCR Detection System (Bio-Rad) and HotStart-IT SYBR Green One-Step qRT-PCR Master Mix Kit (AB science). qRT-PCR primers used for amplification of Nur77, Nor1, KLFs, TM and 18s RNA were: human Nur77 (forward primer: 5'-AAGATCCCTGGCTTTGCTGAGCTG-3', reverse primer: 5'-AGGCCAGGATACTGTCAATCCAGT-3'); human Nor1 (forward primer: 5'-ATAGTCTGAAGGGAGGAA-3', reverse primer: 5'-ATCTTGCGAGTTGGAGGAGAAG-3'); human TM (forward primer: 5'-CTTGCTCATAGGATCTCCATCC-3', reverse primer: 5'-GCGACTTGATCTCCTCATC-3'); human KLF2 (forward primer: , reverse primer:); human KLF4 (forward primer: , reverse primer:); human KLF6 (forward primer: , reverse primer:); human 18s (forward primer: 5'-GTAACCCGTTGAACCCCAT-3', reverse primer: 5'-CTCATCGGTGAGGACG-3'); Mouse TM primer (Forward primer 5'-CCTGTCCTAGTATCCT-3', Reverse primer 5'-TGGTGGTGAGTAC-3'); Mouse GAPDH primer (Forward primer 5'-CGAGTTTCTCGAAAGG-3', Reverse primer 5'-GGGAGTTCATTGAAGGAC-3').
Message RNA Stability Experiment—Actinomycin D (10 μg/ml), an mRNA synthesis inhibitor, was added to cells following the treatments under various experimental conditions. Total RNA was extracted at 0 h, 0.5 h, 1 h, 2 h, 4 h and 6 h after the addition of actinomycin D, and qRT-PCR was then performed. mRNA decay curves were constructed, and the half-life \( (t_{1/2}) \) was calculated from the curves \(^3\) \(^4\) \(^5\).

Electrophoretic Mobility Shift Assay (EMSA) — The oligonucleotides corresponding to the consensus sequence of NF-κB (5′- AGTTGAGGGGACTTTCCCAGGC-3′) was synthesized and labeled with IRDye 700 (IDT). Electrophoretic mobility shift assay (EMSA) were performed with Odyssey® IRDye® 700 infrared dye labelled double-stranded oligonucleotides coupled with the EMSA buffer kit (LI-COR Bioscience, Lincoln, NE, USA) according to manufacturer's instructions. Briefly, labeled probe were incubated with 5 μg nuclear extracts in the binding buffer (100 mM Tris, 500 mM KCl, 10 mM DTT; pH 7.5), Poly(dI•dC) (1 μg/μL in 10 mM Tris, 1 mM EDTA; pH 7.5), 25 mM DTT/2.5% Tween®20 for 30 minutes at room temperature. The probe–protein complexes were separated by electrophoresis in a 4% native polyacrylamide gel containing 50 mM Tris, pH 7.5; 0.38 M glycine; and 2 mM EDTA. The gel was then scanned by Odyssey Imaging System (Li-Cor Biosciences, Lincoln, NE, USA).

Protein C activity assay —HUVECs (2×10⁴ per well) were seeded in a 96-well plate and transduced with Ad-LacZ, Ad-Nur77 or Ad-Nor1 for 48 hr. Cells were washed with PBS and incubated with protein C (80 nM), CaCl₂ (5mM), thrombin (2 nM) in a total volume of 50 μl. 2 hours after incubation at 37 °C, 25 μl of hirudin (4 units/ml) was added to the cells to neutralize thrombin. 10 min after addition of hirudin, 25 μl of chromogenic substrate S-2366 (0.5 mM) was added to each well, and the change in absorbance at 405 nm was measured using a microplate reader every 30 s for 5 min. All of the concentrations listed were final concentrations.

In Vitro Clotting Assay —The assay was performed as described previously \(^6\). HUVECs (2×10⁴ per well) were seeded in a 96-well plate and transduced with Ad-LacZ, Ad-Nur77 or Ad-Nor1 for 48 hr, and then incubated in the absence or presence of TNF-α (20 ng/ml) for another 5 hr. Cells were washed twice with PBS. 100 μl of 37 °C human plasma and 100 μl of 25 mM CaCl₂ were added to each well. Immediately thereafter, this 96-well plate was placed in a microplate reader and read at 405 nm every 20 s for 30 min. An increase in absorbance indicates clot formation. Analysis of kinetic profiles was performed to determine time to reach half-maximal absorbance (\( T_{1/2} \) max).

In vivo thrombosis —Carotid artery thrombosis was induced by photochemical injury as described previously \(^7\). Briefly, mice were anesthetized with sodium pentobarbital (70-90 mg/kg intraperitoneally) and a midline surgical incision was made to expose the right common carotid artery and a Doppler flow probe (Model 0.5 VB; Transonic Systems, Ithaca, NY) was placed under the vessel. Rose Bengal (Sigma-Aldrich, St. Louis, MO, USA) at 50 mg/kg in 0.9% saline was then injected into the tail vein in a 0.12-ml volume. After injection into the tail vein, the right common carotid artery was
transilluminated immediately proximal to the flow probe with a 540 nm green laser (Melles Griot, Carlsbad, CA) from a distance of 6 cm. Blood flow was monitored continuously for 90 minutes from the onset of injury. The time to occlusion was determined after the vessel remained closed with a cessation of blood flow for ≥ 10 minutes. All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee at Thomas Jefferson University.

**Tail-bleeding time**—Bleeding time was measured by a modification of a previously described method. Mice were anesthetized with a mixture of ketamine and xylazine and placed on a 37 °C heating pad. Tails were transected 4 mm from the tip with a scalpel blade. After transection, the tail was immediately placed in a 50-mL falcon tube filled with saline at 37°C, and the time it took for bleeding to stop was measured.

**Hematologic Measurements**—Mice were euthanized for blood collection by cardiac puncture. White blood cell (WBC), hemoglobin and platelet counts were measured using an automated Coulter Counter.

**Statistical Analysis**

All values are expressed as the means ± S.E. Differences between means were analyzed using a two-tailed Student t test. Significant differences were taken at $P<0.05$.

**References:**

antagonists to thrombin and factor viia and activation of protease-activated receptor 1. The Journal of pharmacology and experimental therapeutics. 2004;311:492-501

Supplementary Results

**Supplemental Figure I:** Nur77 increases thrombomodulin mRNA stability in mouse lung microvessel endothelial cells. Mouse lung microvessel endothelial cells were transduced with Ad-LacZ, Ad-Nur77 (A) or Ad-Nor1 (B) at MOI=50. 48 hours after transduction, cells were treated with actinomycin D for 0 h, 0.5 h, 1 h, 2 h, 4 h and 6 h. Cells were harvested for the extraction of total RNAs and qRT-PCR was performed to determine the TM mRNA half-life. The data are representative of 3 independent experiments.
**Supplemental Figure II: Nucleotide sequence of human TM promoter.**

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Supplemental Figure III: Nur77 and Nor1 are involved in VEGF-induced TM expression in HUVECs. A, HUVECs transfected with control siRNA (siCTL), Nur77 siRNA (siNur77), Nor1 siRNA (siNor1) or both. 72 hours after transfection, cells were treated with VEGF (100ng/ml) or vehicle for 1 hour. Expression of Nur77 was determined by qRT-PCR. n=3. *P<0.05 vs siCTL/vehicle; #P<0.05 vs siCTL/VEGF. B, HUVECs transfected with control siRNA (siCTL), Nur77 siRNA (siNur77), Nor1 siRNA (siNor1) or both. 72 hours after transfection, cells were treated with VEGF (100ng/ml) or vehicle for 1 hour. Expression of Nor1 was determined by qRT-PCR. n=3. *P<0.05 vs siCTL/vehicle; #P<0.05 vs siCTL/VEGF. C, HUVECs transfected with control siRNA (siCTL), Nur77 siRNA (siNur77), Nor1 siRNA (siNor1) or both. 72 hours after transfection, cells were treated with VEGF (100ng/ml) or vehicle for 6 hours. Expression of TM was determined by qRT-PCR. n=3. *P<0.05 vs siCTL/vehicle; #P<0.05 vs siCTL/VEGF.

Supplemental Figure IV: Effects of Nur77 and Nor1 on tissue factor expression and NF-κB activation. A, HUVECs were transduced with Ad-LacZ, Ad-Nur77 or Ad-Nor1 (MOI=50). 48 hours after virus transduction, cells were stimulated with 20 ng/ml TNF-α for 3 hours and the expression of tissue factor was determined by qRT-PCR. *P<0.05 vs Ad-LacZ/vehicle; #P<0.05 vs Ad-LacZ/TNF-α. The data are representative of 3 independent experiments. B, HUVECs were transduced with Ad-LacZ, Ad-Nur77 or Ad-Nor1 (MOI=50). 48 hours after virus transduction, cells
were then stimulated with 20 ng/ml TNF-α for 1 hour. Nuclear fraction was isolated for the determination of NF-κB activation by electrophoretic mobility shift assay (EMSA).