Rapid Communication

Kruppel-Like Factor 2 Inhibits Protease Activated Receptor-1 Expression and Thrombin-Mediated Endothelial Activation

Zhiyong Lin, Anne Hamik, Rajan Jain, Ajay Kumar, Mukesh K. Jain

Objective—The serine protease thrombin can dramatically alter endothelial gene expression in a manner that confers a proinflammatory phenotype. Recent studies have identified the Kruppel-like factor 2 (KLF2) as a critical regulator of endothelial gene expression. Herein, we provide evidence that KLF2 inhibits thrombin-mediated endothelial activation via alterations in expression of its principal receptor protease-activated receptor-1 (PAR-1).

Methods and Results—Forced expression of KLF2 in human umbilical vein endothelial cells potently inhibited the ability of thrombin to induce multiple prothrombotic factors (tissue factor, CD40L, plasminogen activator inhibitor-1), cytokines/chemokines (eg, monocyte chemotactic protein-1, interleukin-6 [IL-6], IL-8), and matrix degrading enzymes (eg, matrix metalloproteinases 1, 2, and 9). Mechanistically, KLF2 inhibits PAR-1 expression and, as a consequence, thrombin-mediated nuclear factor κB (NF-κB) nuclear accumulation and DNA binding. Conversely, small interfering RNA–mediated knockdown of KLF2 increases PAR-1 expression and thrombin-mediated induction of NF-κB activation.

Conclusion—These studies identify KLF2 as a novel regulator of PAR-1 expression and thrombin action in endothelial cells. (Arterioscler Thromb Vasc Biol. 2006;26:1185-1189.)

Key Words: Kruppel ■ thrombin ■ PAR-1 ■ transcription ■ endothelial cells

Thrombin is generated by the cleavage of prothrombin at sites of vascular injury and is a key factor involved in the conversion of fibrinogen to fibrin. In addition to regulating the coagulation cascade, thrombin activates a variety of cell types including endothelial cells, smooth muscle cells, leukocytes, and platelets. In endothelial cells, thrombin can alter the expression of multiple factors that collectively confer a proinflammatory phenotype. For example, thrombin can induce the expression of prothrombotic factors such as tissue factor (TF) and plasminogen activator inhibitor-1 (PAI-1). In addition, thrombin can enhance the elaboration of various cytokines (eg, interleukin-6 [IL-6] and IL-8), chemokines (eg, monocyte chemotactic protein-1), and proadhesive molecules (eg, vascular cell adhesion molecule-1) that can augment leukocyte recruitment to the vessel wall. Finally, by inhibiting the expression of factors such as endothelial NO synthase (eNOS) and endothelin-1, thrombin can alter vasomotor tone.

Over the last decade, extensive efforts have been made to delineate the molecular mechanisms underlying the effects of thrombin on cellular function. It is recognized that thrombin exerts its biological function at least in part through a family of G-protein–coupled receptors termed protease-activated receptors (PARs). To date, four PARs have been identified, of which PAR-1, PAR3, and PAR4 are thrombin receptors, whereas PAR2 is activated by trypsin, TF–factor VIIa complex, and factor Xa. In endothelial cells, the predominant receptor responsible for the cellular effects of thrombin is PAR-1.

Kruppel-like factors are a subclass of the zinc-finger family of transcription factors implicated in the regulation of cellular growth and differentiation. Recent studies from our laboratory and others indicate an important role for this family of transcriptional regulators in vascular biology. With respect to endothelial cell biology, Kruppel-like factor 2
(KLF2) has emerged as particularly important. Within the blood vessel wall, KLF2 is expressed exclusively in the endothelial layer.10,11 KLF2 expression is induced by laminar flow and reduced by proinflammatory cytokines.11–13 KLF2 can inhibit the expression of adhesion molecules and prothrombotic factors while inducing antiinflammatory/antiadhesive/antithrombotic factors such as eNOS and thrombomodulin.12,13 By virtue of its differential effects on endothelial gene expression and function, we proposed that KLF2 serves as a key “molecular switch” regulating endothelial function.

In this study, we investigated the role of KLF2 in thrombin-mediated activation of endothelial cells. We find that KLF2 potently inhibits thrombin-mediated proinflammatory activation of endothelial cells. Using both overexpression and small interfering RNA (siRNA)–mediated knockdown approaches, we provide evidence that this effect is through the ability of KLF2 to regulate PAR-1 expression.

Materials and Methods

Cell Culture and Reagents

Human umbilical vein endothelial cells (HUVECs) were acquired from Cambrex Bioscience and cultured in endothelial cell basal medium-2 media according to manufacturer instructions. Human thrombin was obtained from Sigma and used at a final concentration of 2 U/mL for 4 hours in experiments (endotoxin level in medium = 0.01 EU/mL). Endogenous human KLF2 mRNA expression was detected using a 3′UTR fragment of the human KLF2 cDNA. Exogenous KLF2 mRNA expression was detected using a mouse KLF2 cDNA probe. Antibodies recognizing p65, PAR-1, and Ku70 were from Santa Cruz Biotechnology, TF antibody was from American Diagnostica, and α-tubulin antibody was from Sigma. Allophycocyanin (APC)-conjugated anti-mouse antibody was purchased from Caltag. All adenosivlar constructs were generated by the Harvard Gene Therapy Initiative.

Northern and Western Blot Analysis and ELISA Assay

HUVECs were infected with adenovirus encoding green fluorescent protein (Ad-GFP) (control virus [EV]) and Ad-GFP-KLF2 (K2) for 48 hours, exposed to the indicated stimulus, and total RNA or protein harvested for Northern and Western blot as described previously.13 For protein secretion assay, ELISA (Pierce Biotechnology) was performed. Twenty-four hours after infection, HUVECs were starved in 0.5% FBS for 24 hours before treatment with thrombin (2 U/mL) for 4 hours. The supernatants were collected and assessed by ELISA.

siRNA Transfection and Transient Transfection Analysis

Transfection was performed as described previously.13 Forty-eight hours after transfection, cells were treated with or without thrombin for 4 hours and harvested for protein or RNA to for subsequent experiments. Transient transfection was performed as described previously using FuGene13 (Roche). The PAR-1 promoter was kindly provided by Dr C. Tiruppathi (University of Illinois at Chicago).

Gel-Shift Studies

HUVECs were infected with EV or Ad-KLF2 for 48 hours or transfected with siRNA as described above, followed by treatment with thrombin (2 U/mL) for 4 hours. The nuclear extracts were used for gel-shift as described using the nuclear factor κB (NF-κB) consensus binding sites.13

Results

KLF2 Regulates Thrombin-Mediated Endothelial Activation

Recent studies from our laboratory indicate that the reduction in KLF2 expression by stimuli such as tumor necrosis factor-α may be an important means by which cytokines activate endothelial cells.13,14 Thrombin is an important endogenous regulator of inflammation and coagulation. Indeed, treatment of HUVECs with thrombin decreases KLF2 expression (Figure 1A). To gain insight into the effect of KLF2 on thrombin effects on endothelial cell gene expression, we undertook adenosivlar overexpression studies. As shown in Figure 1B and 1C, overexpression of KLF2 (but not the control adenovirus EV) potently inhibited thrombin-mediated induction of TF at both mRNA and protein levels. To gain a more comprehensive understanding of the effects of KLF2 on thrombin-mediated endothelial proinflammatory activation, we assayed for a number of factors implicated in the pathogenesis of vascular disease. As shown in Figure 1D, KLF2 significantly reduced the secretion of cytokines such as IL-6/IL-8, chemokines such as monocyte chemotactic protein-1, and procoagulant factors such as PAI-1 and CD40L. In addition, KLF2 differentially altered the expression of matrix metalloproteinases (MMPs) and their inhibitors (tissue inhibitors of metalloproteinase [TIMPs]), factors that critically regulate tissue remodeling. Specifically, KLF2 reduced the expression of matrix metalloproteinases 1, 2, and 9 while inducing the expression of TIMPs 1 and 2. Finally, we observed no significant effect of platelet-derived growth factor-BB and transforming growth factor-β (TGF-β) secretion in the presence of absence of thrombin (data not shown).

KLF2 Inhibits NF-κB Activation by Thrombin

Thrombin-mediated activation of NF-κB is an important mechanism underlying its proinflammatory effects. We demonstrated previously that in the setting of IL-1β activation of endothelial cells, KLF2 inhibits NF-κB nuclear accumulation or DNA binding. To determine whether similar or distinct mechanisms are operative in the setting of thrombin stimulation, we first performed gel-shift assays. HUVECs were infected with EV or KLF2 (K2), stimulated with IL-1 or thrombin for 4 hours, and nuclear extracts harvested for gel-shift assays. Consistent with our previous observations, KLF2 did not alter NF-κB nuclear accumulation or DNA binding after IL-1β–mediated activation of endothelial cells (Figure 2A, right side; data not shown). However, in marked contrast, NF-κB DNA binding after thrombin activation is strongly reduced. Furthermore, both inhibitor of NF-κBα (IκBα) degradation and p65 nuclear accumulation after thrombin stimulation were reduced in the presence of KLF2 (Figure 2B). These findings strongly indicate a distinct mechanism underlying the ability of KLF2 to inhibit NF-κB function in the setting of thrombin activation.

KLF2 Inhibits Thrombin-Induced Proinflammation Through Inhibition of PAR-1 Expression

The fact that IκBα degradation was altered by KLF2 after thrombin stimulation indicates that a relatively proximal
event in the thrombin signal transduction pathway may be affected. Because thrombin-mediated activation of endothelial cells begins with binding and subsequent proteolytic cleavage of its main receptor PAR-1, we first assessed the effect of KLF2 on PAR-1 levels. As shown in Figure 3A, overexpression of KLF2 reduced PAR-1 mRNA and protein expression in HUVECs. Consistent with these observations, KLF2 also reduced PAR-1 expression on the surfaces of HUVECs (Figure 3B). Conversely, siRNA-mediated knockdown of KLF2 increased PAR-1 expression. Finally, transient transfection studies revealed that KLF2 inhibits PAR-1 promoter activity (Figure 3C). These

Figure 1. KLF2 regulates thrombin-mediated endothelial activation. A, Thrombin inhibits KLF2 mRNA expression. HUVECs were treated with thrombin (2 U/mL) for 4 hours and total RNA harvested and assessed for KLF2 expression by Northern blot. B, Effect of KLF2 on TF expression. HUVECs were infected with the indicated adenovirus at 10 multiplicities of infection (MOI) for 48 hours, stimulated with thrombin for 4 hours, and the expression of TF assessed by Northern (B) and Western (C) analysis. K2 indicates Ad-KLF2. D, KLF2 regulates the secretion of multiple factors involved in thrombin-mediated endothelial activation. Infected HUVECs were treated with thrombin as in B; the supernatants were collected and assessed by ELISA. n=3; *P<0.05; **P<0.005.

Figure 2. KLF2 inhibits NF-κB activation by thrombin. A, KLF2 inhibits NF-κB DNA binding activity. HUVECs were infected with EV and KLF2 adenovirus (K2) for 48 hours, exposed to thrombin or IL-1β for 4 hours, and then gel-shift performed. SS indicated supershift with p65 antibody. B, KLF2 decreases p65 accumulation in the nucleus. Ku70 and tubulin were used for nuclear and cytosolic loading control, respectively.
thrombin-mediated NF-κB (siRNA to KLF2, siKLF2) exhibited a significant increase of nuclear accumulation (top graph) and TF expression (bottom graph). (Figure 3D)

KLF2 inhibits PAR-1 promoter activity. n = 8; *P < 0.005. E, KLF2 deficiency results in augmented thrombin-induced p65 nuclear accumulation (top graph) and TF expression (bottom graph). (Figure 3E)

Collectively, these observations suggest that alteration in KLF2 levels affects PAR-1 expression and thrombin action.

Discussion

The central finding of this study is that KLF2 inhibits thrombin-mediated activation of endothelial cells. This effect is attributable to inhibition of PAR-1 expression and, as a consequence, NF-κB nuclear accumulation and DNA binding. These data identify PAR-1 as a novel target of KLF2 action and may have important implications for endothelial activation as well as other cellular systems in which PAR-1 activation is operative.

In endothelial cells, thrombin elicits a diverse array of biologic responses that induce vascular inflammation, thrombosis, leukocyte recruitment, vasoconstriction, and angiogenesis.1 Thrombin binding and subsequent proteolytic cleavage of its receptor PAR-1 can induce a number of signaling pathways that converge on several transcriptional mediators. From the standpoint of endothelial inflammation, the most critical effector is NF-κB.1 A critical and surprising observation in our studies was the fact that KLF2 inhibits thrombin-mediated NF-κB binding. This is in marked contrast to our observations using IL-1β, in which we observed no effect on NF-κB binding.12 In the latter setting, we found that KLF2 recruited critical coactivators such as p300/CREB-binding protein away from NF-κB and thereby attenuated its ability to activate gene transcription. Because thrombin-induced NF-κB nuclear accumulation was also reduced by KLF2 (Figure 2), we reasoned that a proximal event must account for this effect. Indeed, as shown in Figure 3, KLF2 is an endogenous regulator of the key thrombin receptor PAR-1, the most proximal mediator of thrombin signaling in endothelial cells. This is an important observation in light of the paucity of information regarding the transcriptional regulation of PAR-1. Studies to date indicate an important role for Sp1 as an inducer of PAR-1 promoter activity.15 In addition, the factor activator protein-2 has been identified as an inhibitor of PAR-1 expression and promoter activity.15 Our studies show that KLF2 can inhibit PAR-1 promoter activity, supporting a direct role of KLF2 in regulating PAR-1 expression. Whether this occurs directly as a consequence of KLF2 binding to the PAR-1 promoter or through displacement of an activator is the subject of ongoing studies. Finally, although our observations implicate the KLF2 regulation of PAR1 as important in regulating the inflammatory effects of thrombin, we cannot exclude the possibility that other mechanisms may be operative.

Finally, the ability of KLF2 to inhibit thrombin action may have broader vascular implications than its effect on prothrombotic state of endothelial cells. For example, previous studies indicate that thrombin can induce angiogenesis and vasoconstriction. The former is thought to occur via induction of the vascular endothelial growth factor (VEGF) receptor VEGFR2,16 whereas the latter effect results from alterations in eNOS and endothelin-1 expression.17,18 We have shown previously that KLF2 can inhibit angiogenesis specifically via reduction of VEGFR2.19 Furthermore, KLF2 can potentially induce eNOS12,13 and inhibit endothelin-1.20 Whether KLF2

Figure 3. KLF2 regulates thrombin-mediated endothelial function through inhibiting PAR-1. A, KLF2 regulates PAR-1 expression. HUVECs were infected with 10 multiplicities of infection of EV or KLF2 adenovirus (K2) or transfected with nonspecific siRNA (NS) or KLF2-specific siRNA. Forty-eight hours later, total RNA and protein were harvested for PAR-1 expression assessment. Exo-KLF2 and endo-KLF2 refer to exogenously expressed mouse KLF2 and endogenous human KLF2, respectively. B, KLF2 knockdown increases NF-κB DNA binding. HUVECs were transfected as in A; followed by treatment with thrombin, and gel-shift assay was performed. SS indicated supershift with p65 antibody. C, KLF2 reduced HUVEC surface expression of PAR1. Infected HUVECs were incubated with PAR-1 antibody followed by a secondary antibody conjugated with allophycocyanin and assessed by fluorescence-activated cell sorter analysis. D, KLF2 inhibits PAR-1 promoter activity. n = 8; *P < 0.005. E, KLF2 deficiency results in augmented thrombin-induced p65 nuclear accumulation (top graph) and TF expression (bottom graph).
can affect these additional cellular processes in which PAR-1 plays an important role is the subject of ongoing studies.

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