Thioredoxin Interacting Protein Promotes Endothelial Cell Inflammation in Response to Disturbed Flow by Increasing Leukocyte Adhesion and Repressing Kruppel-Like Factor 2

Xiao-Qun Wang, Patrizia Nigro, Cameron World, Keigi Fujiwara, Chen Yan, Bradford C. Berk

**Rationale:** Endothelial cells (EC) at regions exposed to disturbed flow (d-flow) are predisposed to inflammation and the subsequent development of atherosclerosis. We previously showed that thioredoxin interacting protein (TXNIP) was required for tumor necrosis factor-mediated expression of vascular cell adhesion molecule-1.

**Objective:** We sought to investigate the role of TXNIP in d-flow-induced cell adhesion molecule expression and leukocyte interaction with vessels, and the mechanisms by which TXNIP suppresses athero-protective gene expression.

**Methods and Results:** Using en face staining of mouse aorta, we found a dramatic increase of TXNIP in EC at sites exposed to d-flow as compared to steady flow. EC-specific TXNIP (EC-TXNIP) knockout mice showed significant decreases in vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 mRNA expression in the d-flow regions of mouse aorta. Intravital microscopy of mesenteric venules showed that leukocyte rolling time was decreased, whereas rolling velocity was increased significantly in EC-TXNIP knockout mice. In vitro experiments using a cutout flow chamber to generate varying flow patterns showed that increased TXNIP was required for d-flow-induced EC-monocyte adhesion. Furthermore, we found that the expression of Kruppel-like factor 2, a key anti-inflammatory transcription factor in EC, was inhibited by TXNIP. Luciferase and chromatin immunoprecipitation assays showed that TXNIP was present within a repressing complex on the Kruppel-like factor 2 promoter.

**Conclusions:** These data demonstrate the essential role for TXNIP in mediating EC-leukocyte adhesion under d-flow, as well as define a novel mechanism by which TXNIP acts as a transcriptional corepressor to regulate Kruppel-like factor 2-dependent gene expression.  (**Circ Res. 2012;110:560-568.**)

**Key Words:** TXNIP ■ KLF2 ■ disturbed flow ■ cell adhesion molecules

Atherosclerotic lesions occur in a nonrandom pattern characterized by preferential initiation at sites of disturbed blood flow (d-flow) as compared to regions of steady flow (s-flow). Specifically, atherosclerosis is prominent in d-flow regions such as the lesser curvature of the aortic arch, flow dividers, and branch points. Fluid shear stress, the frictional force generated by blood flow, mechanically acts on endothelial cells (EC) in the vasculature and regulates EC functions. Physiological s-flow present within straight segments of the arterial tree, is of high shear stress (>10 dyn/cm²) and athero-protective. In contrast, d-flow, characterized by a nonunidirectional, nonsteady flow pattern with a low magnitude of shear stress (<5 dyn/cm²), promotes endothelial dysfunction by increasing intracellular reactive oxygen species (ROS) production, endothelial permeability, and the expression of cell adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1). The induction of these cell adhesion molecules in response to d-flow leads to increased binding of circulating leukocytes, which transmigrate across the EC, create chronic inflammation, and promote atherogenesis.

The mechanisms by which s-flow increases athero-protective gene expression have been shown to be mediated...
primarily (proximately 70%) by a MEK5-ERK5-KLF2 pathway.11 Kruppel-like factor 2 (KLF2) is a key anti-inflammatory transcription factor in EC11,12 and is exclusively induced by s-flow but not by d-flow.13 Specifically, KLF2 has been shown to enhance the expression of anti-inflammatory genes such as endothelial nitric oxide synthase (eNOS) on one hand, and inhibit the induction of cell adhesion molecules by cytokines on the other.12

In contrast, less is known regarding specific d-flow mechanisms. Our laboratory and others have shown that d-flow is associated with increased PKCζ activity14 and p53 activation.15 Previously, we reported that EC thioredoxin interacting protein (TXNIP, also termed as TBP-2) was reduced by s-flow, thereby increasing thioredoxin activity and suppressing EC inflammation in response to tumor necrosis factor-α via inhibiting JNK and p38 signaling pathways.16 TXNIP has multiple functions in EC by regulating metabolism,17 cell growth,18 and inflammation.19 TXNIP is a member of α-arrestin family20 and acts as a scaffold for several proteins besides thioredoxin,21 including NLRP319 and histone deacetylase (HDAC).18,22 However, the role of TXNIP in d-flow-induced EC dysfunction has not been investigated. In the present study, we show that the increase of TXNIP is required for d-flow–induced cell adhesion molecule expression and leukocyte adhesion. Furthermore, we show that TXNIP promotes EC dysfunction by acting as a transcriptional corepressor of the KLF2 promoter to inhibit KLF2 expression and its downstream signaling.

Methods

An expanded Methods section is provided in the online-only Data Supplement.

Animals

All animal experiments were conducted in accordance with experimental protocols that were approved by the University Committee on Animal Resources of the University of Rochester. To generate EC-specific TXNIP knockout mice, female TXNIPflox/flox mice (made by Dr Roger Davis, San Diego State University) were crossed with male Cre recombinase transgenic mice under the control of endothelial-specific receptor tyrosine kinase 2 (Tie2) promoter (Tie2-Cre+, purchased from Jackson Laboratory, #4128) to generate heterozygous Tie2-Cre+ TXNIPflox/+ mice for breeding. Then homozygous EC-specific TXNIP knockout mice (Tie2-Cre+- TXNIPflox/flox) were obtained and the Cre negative littermates (Tie2-Cre- TXNIPflox/flox) were used as controls. Animals were maintained under pathogen-free conditions of the Aab Cardiovascular Research Institute of the University of Rochester.

Cell Culture

Human umbilical vein endothelial cells (HUVEC) were cultured as detailed in the online-only Data Supplement.

Plasmid and siRNA Oligonucleotide Transfection

HUVEC were transfected with TXNIP-green fluorescent protein, TXNIP specific siRNA, or luciferase reporter gene constructs as detailed in the online-only Data Supplement.

Flow Apparatus

Confluent HUVEC cultured in 35-mm dishes were exposed to s- and d-flow at 37°C in a humidified 5% CO2 tissue culture incubator using a parallel-plate flow chamber (GlycoTech, Gaithersburg, MD). Cutouts (1 mm×1 mm) were made on both sides of the silastic gasket to create shear gradients and vortices within the flow path.23 When culture medium was circulated through the chamber using a peristaltic pump (EP-1 Econo Pump, Bio-Rad, Hercules, CA), cells in the main, central part of the flow chamber were exposed to s-flow (12 dyn/cm2) while small disturbed flow areas were created within the cutouts. Shear stress in the s-flow region was calculated by the formula \( \mu Q / h b^2 \), where \( Q \) is the flow rate, \( \mu \) is the fluid viscosity, \( w \) is the width of the channel, and \( h \) is the thickness of the gasket. After 24 hours, EC in the cutout area exhibited polygonal cell shapes, whereas cells in the middle part of chamber were aligned parallel to the direction of flow. To expose a large amount of cells to flow for biochemical studies, we used a cone-and-plate flow apparatus as described previously.15,16 Confluent HUVEC cultured in 100-mm dishes were exposed to s-flow at 12 dyn/cm2 by using smooth cones,16 or exposed to d-flow by using grooved cones at 5 rpm (gives 0.6 dyn/cm2 of s-flow) for the indicated times.15,16 The shear stress imposed on the surface of the plate was calculated by the formula \( \mu Q / \omega b^2 \) where \( \omega \) is rotation speed, \( \mu \) is the fluid viscosity, and \( \theta \) is the angle of the cone.

Luciferase Assay

The luciferase activities of the KLF2 promoter and NF-κB binding site were assayed as detailed in the online-only Data Supplement.

En Face Immunofluorescence Staining

En face immunofluorescence staining of mouse aorta to compare TXNIP expression in different regions (s-flow versus d-flow) was performed as detailed in the online-only Data Supplement.

In Vitro THP-1 Monocyte Adhesion Assay

EC-monocyte adhesion was assayed by coincubating fluorescently labeled THP-1 monocytes with HUVEC as detailed in the online-only Data Supplement.

Intravital Microscopy

Age-matched male EC-TXNIP knockout and control mice (3–5 months) were anesthetized by intraperitoneal injection of ketamine/
Xylazine cocktail (0.13/0.0088 mg/g body weight). Leukocytes were labeled by retro-orbital injection of 50 μL 0.05% Rhodamine 6G. Then mesenteric venules were exteriorized and rolling leukocytes were monitored by inverted fluorescent microscope (Nikon Eclipse Ti, Nikon) equipped with a stage warmer (Thermo Plate, Tokai Hit), a QuantEM:512SC camera (Photometrics, Tucson, AZ), and a Nikon S Plan Fluor ELWD 20× lens. Image-Pro V6.2 was used to automatically track moving leukocytes. Then rolling time and velocity were calculated.

Real-Time Quantitative Polymerase Chain Reaction
Relative mRNA expression of VCAM-1, ICAM-1, and KLF2 was quantified by real-time polymerase chain reaction (PCR) as detailed in the online-only Data Supplement.

Western Blot Analysis
Protein expression of TXNIP, VCAM-1, ICAM-1, KLF2, and eNOS was quantified by Western blot as detailed in the online-only Data Supplement.

Chromatin Immunoprecipitation Assay
Chromatin immunoprecipitation assay (ChIP) assay to investigate the association of TXNIP with the KLF2 promoter was performed as detailed in the online-only Data Supplement.

Statistical Analysis
Data are shown as mean±SD from 3 to 7 independent experiments. Leukocyte rolling events were compared by using Mann–Whitney U test. Other differences were analyzed by two-tailed Student t-test. Probability values <0.05 were considered statistically significant.

Results
Disturbed Flow Increases TXNIP Expression in EC
To study the effect of d-flow on TXNIP expression, we performed en face immunofluorescence staining on mouse aorta to compare EC TXNIP expression levels under different flow patterns. We found TXNIP was dramatically increased in d-flow regions, including the lesser curvature of the aortic arch (area L in Figure 1A and 1B) and the branch points of outflow tracts (bottom panel in Figure 1A, Online Figure I, and the corresponding blue area in Figure 1B), when compared with nearby athero-protective s-flow regions, such as the greater curvature (area G in Figure 1A and 1B) and the main segment of the thoracic aorta (bottom panel in Figure 1A) both from C57BL/6 wild type and ApoE−/− mice. To confirm the increase of TXNIP expression by d-flow in vitro, we used a parallel flow chamber with cutouts (Figure 1C) that creates adjacently located d- and s-flow areas. After 24 hours of flow treatment (12 dyn/cm² in the s-flow region), a greater expression of TXNIP in HUVEC was observed in the d-flow cutout area than in the s-flow region (Figure 1D and 1E). TXNIP expression was assayed by immunofluorescence. EC morphology is shown by VE-Cadherin staining.
TXNIP is Required for D-Flow-Induced Cell Adhesion Molecule Expression and EC-Monocyte Adhesion

A key reason that d-flow promotes atherosclerosis is the pronounced expression of cell adhesion molecules on the endothelial surface (Online Figure III), which facilitates leukocyte recruitment and vascular inflammation. To investigate whether TXNIP plays a role in this process, we established EC-specific TXNIP knockout (EC-TXNIP KO) mice by using Cre/LoxP strategy. We generated Tie2-Cre/TXNIPflox/flox mice as knockout mice and Tie2-Cre-TXNIPflox/flox littermates as controls (Online Figure IV). Then real-time PCR was performed to determine if TXNIP regulates cell adhesion molecule expression. We found that in control animals, VCAM-1 (9.75/2.99-fold) and ICAM-1 (2.15/0.23-fold) mRNA expression levels were markedly increased in the lesser curvature of the aortic arch where the flow pattern is disturbed, as compared to the thoracic aorta where the majority of EC are under s-flow (Figure 2A and 2B). Importantly, the increased expression of these cell adhesion molecules in the d-flow area was significantly inhibited in EC-TXNIP KO mice (Figure 2A and 2B). In vitro knockdown of TXNIP also showed dramatic decreases in VCAM-1 and ICAM-1 expression under d-flow (Figure 2C).

Next, we performed an in vitro adhesion assay to show that TXNIP plays a role in the enhanced EC-leukocyte adhesion under d-flow. HUVEC cultured in the cutout chamber were pretreated with either scramble control or TXNIP-specific siRNA for 48 hours. Then EC were exposed to d-flow or kept at static condition for an additional 24 hours. Protein expression of VCAM-1, ICAM-1, and TXNIP was analyzed by Western blot.

TXNIP Deficiency in EC Reduces Leukocyte-Endothelium Adhesiveness

Leukocyte adhesion to endothelium is one of the earliest steps in atherogenesis, which involves the initial attachment of...
circuiting leukocytes, capture, and subsequent firm adherence. Similar as the d-flow area in the conduit artery, the venous system is exposed to low shear stress ranging from 1 to 6 dyn/cm². Moreover, intravital microscopy of mesenteric venules is an established method to analyze leukocyte-endothelium interactions. Thus, to study the role of TXNIP in leukocyte adhesion in vivo, we performed intravital microscopy to quantify leukocyte rolling time and rolling velocity in mesenteric venules (n=7) from control (Tie2-Cre⁺ TXNIPfloxflox) and EC-TXNIP KO (Tie2-Cre⁺ TXNIPfloxflox) mice. Leukocyte movement was monitored in real time (Figure 4A). Compared with control animals, significant decreases in rolling time (Figure 4B) and marked increases in rolling velocity (Figure 4C) of fluorescently labeled leukocytes were observed in EC-TXNIP KO mice. Especially, the number of leukocytes with long rolling time (5 s–20 s) was substantially decreased in EC-TXNIP KO mice (Figure 4D). In addition, no significant difference in EC-leukocyte adhesion was detected between Tie2-Cre⁺ TXNIPfloxflox and Tie2-Cre⁺ transgenic mice (data not shown), which confirms that the decrease of endothelial adhesiveness in EC-TXNIP KO mice is due to the depletion of TXNIP but not Cre toxicity. Comparison of the 2 strains showed no changes in systolic blood pressure (Online Figure VIA), heart rate (Online Figure VIB), and TXNIP expression in peripheral blood mononuclear cells (Online Figure VIB). Taken together, these results clearly show that TXNIP deficiency reduces leukocyte adhesion to endothelium, which confirms the role of TXNIP in modulating the expression of proinflammatory cell adhesion molecules.

TXNIP Suppresses KLF2 Expression and Promoter Activity

KLF2 has been reported to be an important anti-inflammatory transcription factor in EC. It is highly induced by s-flow but not by d-flow, and antagonizes the induction of cell adhesion molecules in response to tumor necrosis factor-α and IL-1β in EC. Thus, we next hypothesized that KLF2 is regulated by TXNIP in EC in a flow-dependent manner. We found KLF2 expression was decreased by d-flow (Figure 5A). Moreover, overexpression of KLF2 virtually abolished the d-flow–induced VCAM-1 and ICAM-1 expression (Figure 5A), suggesting that the inhibition of KLF2 by d-flow is required for cell adhesion molecule expression.

Next, we found that KLF2 mRNA levels in the lesser curvature of the aortic arch (d-flow region) were significantly increased in EC-TXNIP KO mice by using real-time PCR (Figure 5B). Furthermore, knockdown of TXNIP in vitro increased KLF2 protein levels under both static condition and d-flow (Figure 5C). TXNIP has been previously reported to prevent NF-κB activation. We also found that the d-flow–induced NF-κB activation was completely abolished after TXNIP knockdown (Online Figure VIII). On the other hand, overexpression of TXNIP significantly reduced KLF2 protein (Figure 5D) and mRNA (Figure 5E) expression levels under both static condition and s-flow. In addition, the expression of eNOS, a downstream target of KLF2 in EC, was also decreased by TXNIP overexpression (Online Figure IXA).

To establish the mechanism by which TXNIP inhibits KLF2 expression, we studied the effect of TXNIP on KLF2 promoter activity by using luciferase assay. TXNIP overexpression significantly decreased the activity of both the −924 bp full-length promoter and the −157 bp fragment, whereas a further deletion of the promoter (−78 bp construct) resulted in complete loss of inhibition (Figure 6A). These data suggest that the TXNIP-mediated repression occurs through a region from −157 bp to −78 bp of the KLF2 promoter. The −157 bp promoter fragment was previously reported to be responsible for the transactivation of KLF2 by s-flow. Thus, we further investigated whether the inhibition persisted after...
s-flow exposure. We detected a 4.13±0.13-fold increase for the −924 bp promoter construct (Figure 6B) and a 2.61±0.11-fold increase for the −157 bp promoter construct (Figure 6C) in luciferase activity after s-flow treatment, whereas the increase of promoter activity by s-flow was significantly repressed in the presence of TXNIP overexpression for both promoters. Taken together, these results suggest a role for TXNIP as a corepressor of the KLF2 promoter, thereby inhibiting KLF2 expression and its downstream signaling. These data are consistent with the reports that TXNIP associates with transcriptional repressors such as HDAC1 and HDAC3.18,22

Discussion

The major findings of the present study are that TXNIP expression is increased by d-flow, inhibits KLF2 expression, and promotes endothelial-leukocyte adhesion; thereby initi-
biochemical analysis of the promoter sequence and a literature survey demonstrates that the association with the KLF2 promoter is critically important in understanding how TXNIP regulates KLF2 expression. In addition, the ChIP assay data illustrate the mechanism by which TXNIP regulates KLF2 promoter activity, providing a schematic representation showing the mechanism by which TXNIP regulates KLF2 promoter activity. Letter x indicates an unknown factor.

Although TXNIP appears to be a common mediator for regulation of s-flow and d-flow gene expression, the mechanisms by which flow regulates TXNIP and KLF2 expression clearly differ. KLF2 expression is regulated by several transcription factors, including MEF2C,28 PLZF,29 and P30030 or by miRNA post-transcriptionally.31 Using in situ hybridization, Dekker et al showed that KLF2 is exclusively induced by s-flow but not d-flow.13 Our study shows a novel mechanism by which KLF2 is differentially regulated that involves transcription corepression by d-flow. Several mechanisms have been shown to regulate TXNIP expression. To date, these fall into either metabolic or redox-sensitive pathways. For example, the MondoA:Mlx dimer transcription factors promote TXNIP expression by activating a carbohydrate response element on the TXNIP promoter.32 Among the pathways responsive to oxidative stress, both hyperglycemia and d-flow increase intracellular reactive oxygen species levels in EC by activating NAD(P)H oxidase,7,33,34 raising the possibility that a similar mechanism may be responsible for d-flow-induced TXNIP. In addition, Nrf2, an important shear stress-responsive transcription factor regulating redox-sensitive responses,35 has been shown to significantly increase TXNIP expression.36 The roles of MondoA:Mlx and Nrf2 as mediators for TXNIP induction by d-flow need further elucidation.

Our findings have several potential clinical implications. First, as shown by intravital microscopy (Figure 4), we demonstrated that TXNIP is required for leukocyte interaction with vessel walls, suggesting it is a novel target for preventing leukocyte recruitment. Second, the increase of TXNIP in response to d-flow promotes cell adhesion molecule expression; conversely, this effect is inhibited by overexpression of KLF2. This establishes a counterregulatory role for TXNIP and KLF2, which suggests that a strategy to increase KLF2 or decrease TXNIP should provide therapeutic benefits. Finally, given that diabetes is associated with high inflammation (Online Figure X). The ChIP assay data show the mechanism for TXNIP regulation of KLF2 involves the association with the KLF2 promoter. In addition, an important feature of the current study is that we clearly demonstrate by 2 approaches that d-flow athero-prone sites exhibit a dramatic increase of TXNIP expression. First, in vitro studies of s-flow and d-flow using a cutout chamber under identical tissue culture conditions reproduce the in vivo results.23 These results lead to the concept that TXNIP is a mechanosensitive switch for EC inflammation by inhibiting athero-protective pathways downstream of KLF2 and activating athero-promoting gene expression.

We propose that TXNIP is a very important regulator of the genetic programs present under s-flow and d-flow by regulation of KLF2. It is likely that s-flow inhibits TXNIP interaction with the KLF2 promoter by 2 mechanisms. First, total TXNIP expression in EC is decreased by s-flow in a chronic manner.16 Second, s-flow may maintain TXNIP in a cytosolic location that prevents effects on gene transcription. In contrast, d-flow dramatically increases TXNIP expression in the nucleus, which may lead to increased interactions with transcriptional repressors, such as HDAC1 and HDAC3.18,22 Thus, TXNIP seems to act as a corepressor by recruiting transcriptional repressors to the transcription factors that are responsible for KLF2 transactivation (Figure 7F). Furthermore, we established that the activation of the KLF2 promoter in response to s-flow was virtually abolished by TXNIP overexpression, and a small fragment of the KLF2 promoter (−157 bp to −78 bp) was responsible (Figure 6A). Analysis of the promoter sequence and a literature survey failed to yield any known binding motif or associated transcription factor. Identification of the precise mechanism of TXNIP corepression will be an important future goal.
TXNIP expression, therapies that decrease TXNIP would be predicted to be useful for reducing complications in diabetics such as accelerated atherosclerosis.37,38

Acknowledgments

We thank Craig Morrell for the support of intravital microscopy, Kyung-Sun Heo for the support of en face immunofluorescence staining, and Amy Mohan, Christine Christie, and Alison Hobbins for technical assistance.

Sources of Funding

This work was supported by National Institutes of Health Grants HL077789 and HL106158 (to B.C.B. and to C.Y.).

Disclosures

None.

References


### Novelty and Significance

**What Is Known?**

- Thioredoxin interacting protein (TXNIP), a scaffold protein that is a member of the α-arrestin family, is required for tumor necrosis factor-induced endothelial cell (EC) inflammation.
- Disturbed flow (d-flow) leads to EC inflammation and the development of atherosclerosis by increasing cell adhesion molecule expression and EC-leukocyte adhesion.
- Steady flow (s-flow) decreases EC TXNIP expression.
- Kruppel-like factor 2 (KLF2), a key anti-inflammatory transcription factor in EC, is highly induced by s-flow, but not by d-flow.

**What New Information Does This Article Contribute?**

- Enhanced TXNIP expression is responsible for d-flow-induced cell adhesion molecule expression and EC-leukocyte adhesion.
- TXNIP acts as a transcriptional corepressor of the KLF2 promoter, thereby inhibiting KLF2 expression and its downstream signaling.
- TXNIP-KLF2 promoter complex is increased by d-flow and decreased by s-flow.

Atherosclerotic lesions preferentially initiate in the d-flow areas of the arterial tree, such as the lesser curvature of the aortic arch and the branch points of outflow tracts. However, the mechanism by which d-flow promotes EC inflammation is not clear. By using en face staining of mouse aorta in vivo and cutout flow chamber in vitro, we showed that TXNIP is a mechanosensitive gene that is dramatically increased by d-flow. Importantly, the increase of TXNIP is required for the enhanced expression of cell adhesion molecules and EC-leukocyte adhesion under d-flow. Specifically, TXNIP promotes EC inflammation by acting as a transcriptional corepressor of the KLF2 promoter, thereby inhibiting KLF2-dependent anti-inflammatory pathways. These findings provide new insight into the role of TXNIP as a mechanosensitive switch for EC inflammation by inhibiting athero-protective pathways downstream of KLF2, and activating athero-promoting gene expression.
Thioredoxin Interacting Protein Promotes Endothelial Cell Inflammation in Response to Disturbed Flow by Increasing Leukocyte Adhesion and Repressing Kruppel-Like Factor 2
Xiao-Qun Wang, Patrizia Nigro, Cameron World, Keigi Fujiwara, Chen Yan and Bradford C. Berk

*Circ Res.* 2012;110:560-568; originally published online January 19, 2012;
doi: 10.1161/CIRCRESAHA.111.256362

*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/110/4/560

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2012/01/17/CIRCRESAHA.111.256362.DC1

**Permissions:** Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation Research* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

**Reprints:** Information about reprints can be found online at:
http://www.lww.com/reprints

**Subscriptions:** Information about subscribing to *Circulation Research* is online at:
http://circres.ahajournals.org/subscriptions/
SUPPLEMENTAL MATERIAL

Detailed Methods

Plasmids

TXNIP-GFP was obtained from Addgene (Addgene plasmid 18758). Human KLF2 cDNA was purchased from Origene (#SC127849, Rockville, MD). Both of the -924 bp and -157 bp KLF2 promoter reporter gene constructs were gifts from Dr Jerry Lingrel (University of Cincinnati). NF-κB luciferase reporter gene construct was a gift from Dr Chen Yan (University of Rochester).

Antibodies

Rabbit anti-TXNIP (VDUP1, C-term, #403700) and mouse anti-α-tubulin (#322500) were purchased from Invitrogen (Carlsbad, CA). Rabbit anti-VCAM-1 (H-276, #SC-8304), mouse anti-ICAM-1 (G-5, #SC-8439), mouse anti-VE-Cadherin (F-8, #SC-9989), and goat anti-KLF2 (N-13, #SC-18690) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rat anti-VE-Cadherin (#322500) and rabbit anti-eNOS (#610298) were purchased from BD Biosciences Pharmingen (San Diego, CA). Rabbit anti-Lamin A/C (#2032) was purchased from Cell Signaling Technology (Beverly, MA).

Cell culture

HUVEC were isolated from human umbilical veins and seeded onto 0.2% gelatin precoated culture dishes maintained in Medium 200 (Cascade Biologics, Portland, OR) with low-serum growth supplement (LSGS; Invitrogen, Carlsbad, CA), 5% fetal bovine serum (FBS), 100 μg/ml streptomycin and 100 IU/ml penicillin at 37°C in a humidified atmosphere of 95% air and 5% CO2 as previously described.1 HUVEC were used at passage 4 for all the experiments. THP-1 monocytes were grown in RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 10% FBS, 100 μg/ml streptomycin and 100 IU/ml penicillin at 37°C in a humidified atmosphere of 95% air and 5% CO2.

Plasmid and siRNA oligonucleotide transfection

HUVEC at greater than 90% confluence in 100-mm dishes were used for transfection. To overexpress TXNIP or KLF2 in EC, Lipofectamine 2000 (6 μl; Invitrogen, Carlsbad, CA) was mixed with Opti-MEM (500 μl; Invitrogen, Carlsbad, CA), and then cDNA (3 μg) diluted in 500 μl Opti-MEM was added to the solution, mixed gently, and incubated at room temperature for 20 min. A total of 1 ml of this mixture was added to HUVEC in 4 ml Opti-MEM and incubated for 3 h. Then medium was changed back to M200 medium and cells were harvested after 24 h. To knockdown TXNIP in HUVEC, we transfected cells with TXNIP-specific siRNA (#J-010814-05, Dharmacon, Lafayette, CO) by using Lipofectamne 2000 and Opti-MEM following manufacturer’s protocol.

Luciferase assay

HUVEC were transiently co-transfected with luciferase reporter gene constructs and β-galactosidase with Lipofectamine 2000 by using the similar transfection scheme as described above. For flow studies, transfected cells were incubated overnight at 37°C for 18h and then exposed to s-flow (6 h) or d-flow (24 h) by using the cone and plate flow apparatus. To measure luciferase activity, transfected HUVEC were washed twice with phosphate buffered saline (PBS, lysed in Passive Lysis Buffer (#E194A, Promega, Madison, WI), and assayed by using a Luciferase Assay System (#E1501, Promega, Madison, WI) and a luminometer (BD Monolight 3010, BD Biosciences Pharmingen, San Diego, CA). Luciferase activity was normalized to β-galactosidase activity to correct for differences in transfection efficiency.

Immunofluorescence staining

Treated HUVEC in 35mm-dishes were fixed in 4% paraformaldehyde in PBS (pH 7.4) for 15 min, permeabilized with 0.1% Triton X-100 in PBS for 5 min, and blocked with 10% normal goat serum in
PBS containing 0.5% Tween-20 for 1 h at room temperature. Then cells were stained with 5 μg/ml rabbit anti-TXNIP and mouse anti-VE-Cadherin overnight at 4°C in the blocking solution. Cells were then rinsed with 0.5% Tween-20 in PBS 3 times and incubated with the fluorescence-conjugated secondary antibodies (1:1000 dilution, Alex Fluor 546 and 488, respectively) for 1 h at room temperature. After another 3 rinses with the washing solution, images were acquired using an inverted epi-fluorescence microscope (IX50, Olympus) equipped with a charge-coupled device camera (Spot; Diagnostic Instruments, Inc) with Acroplan water 40× (N.A. 0.8) or 60× (N.A. 0.9) objective lens.

**En face immunofluorescence staining**

Immunofluorescence staining of mouse aortic EC was performed as described previously with several modifications. Briefly, 12-week-old C57BL/6 and ApoE−/− mice were anesthetized with ketamine/xylazine cocktail (0.13/0.0088 mg/g body weight). Then we cut the jugular vein and perfused the arterial tree with saline containing 40 USPU/ml heparin from left ventricle for 5 min, followed by perfusion of pre-chilled 4% paraformaldehyde in PBS (pH 7.4) for 10 min. Subsequently, the whole aorta was dissected from iliac bifurcation to the heart, cut open longitudinally, permeabilized with 0.1% Triton X-100 in PBS for 10 min and blocked with 10% normal goat serum in Tris-buffered saline (TBS) containing 2.5% Tween-20 for 1 h at room temperature. Next, aortas were incubated with 5 μg/ml rabbit anti-TXNIP or 2 μg/ml rabbit anti-VCAM-1, and 5 μg/ml rat anti-VE-Cadherin in the blocking buffer overnight at 4°C. After rinsing with washing solution (TBS containing 2.5% Tween-20) 3 times, fluorescence-conjugated secondary antibodies (1:1000 dilution, Alexa Fluor 546 labeled anti-rabbit IgG and Alexa Fluor 488 labeled anti-rat IgG, respectively) were applied for 1 h at room temperature. Finally, after another 3 rinses in the washing solution, aortas were mounted in the ProLong antifade reagent (Invitrogen, Eugene, OR). Aortas were examined by a laser-scanning confocal microscope (FV-1000 mounted on IX81, Olympus) with UPlanSApo 20× or UPlanFL N 40x lens. Image analysis to quantify VCAM-1 membrane localization was performed in Image-Pro V6.2. Briefly, color intensities for both green (VE-Cadherin) and red (VCAM-1) channels from the desired region in the en face images were obtained by using Image-Pro. Then, the relative color intensity for each channel was plotted. Membrane-localized VCAM-1 was considered to be the overlapped area from both green and red channels.

**In vitro THP-1 monocyte adhesion assay**

Human monocyte leukemia cell line THP-1 was labeled for 30 min at 37°C with 10 μmol/L BCECF-AM (#B1170, Molecular Probes/Invitrogen, Eugene, OR) in RPMI 1640 medium, and washed three times with PBS. Labeled THP-1 monocytes (10^6 cells/ml) in M200 medium were added to monolayers of HUVEC and incubated for 40 min. Nonadherent THP-1 cells were removed by washing the culture 4 times with PBS. Cells were then stained with rhodamine-conjugated phalloidin (#R415, Invitrogen, Eugene, OR) and DAPI (#D1306, Invitrogen, Eugene, OR) to show the cell morphology and the nuclei. Attached cells were then observed by an inverted fluorescent microscope (IX50, Olympus) with an Acroplan 40× lens. EC-monocyte adhesion was quantified by calculating the percentage of EC-associated THP-1 monocytes.

**Blood pressure**

Systolic blood pressure and heart rate were measured using a non-invasive tail-cuff method (plethysmography; Visitech System, Apex, NC).

**Peripheral blood mononuclear cells (PBMC) isolation**

Mouse PBMC were isolated by Optiprep density gradient medium (Nycomed, Oslo, Norway) following manufacturer’s protocol. Briefly, diluted blood was layered on a 1.077 g/ml density barrier. After centrifugation at 700g for 20 min, mononuclear cells at interphase were harvested using a pipette.

**Subcellular fractionation**
Nuclear and cytosolic protein was isolated as described previously. In brief, treated HUVEC were washed and incubated with ice-cold hypotonic lysis buffer (10 mM HEPES, 1.5 mM MgCl$_2$, 5 mM KCl, 1 mM DTT, and protease inhibitors) for 10 min. Cells were then scraped and homogenized with 20 strokes of a Dounce homogenizer. Homogenates were centrifuged at 700 g for 3 min to pellet nuclei. The supernatants were kept as cytosolic fraction. Nuclear pellets were resuspended in nuclei lysis buffer (50 mM Tris-Cl, 10 mM EDTA, 1% SDS, and protease inhibitors) as nuclear fraction.

**Real-time quantitative polymerase chain reaction (PCR)**

Total RNA was extracted using a TRIzol reagent (Invitrogen, Carlsbad, CA). For reverse transcription, 1 µg of total RNA was converted into first strand complementary DNA (cDNA) in a 20 µl reaction volume using a reverse transcription kit (#A3500, Promega, Madison, WI) following the manufacturer’s instruction. Quantitative real-time PCR was then performed using an ABI 7900HT real-time PCR thermal cycler (Applied Biosystems, Foster City, CA). The comparative cycle threshold method was used to determine the relative mRNA expression of target genes after normalization to housekeeping gene GAPDH. Following primers were used: 5’-GAACCCAAAACAGAGCAG-3’ for mouse VCAM-1-F and 5’-GTATCCATCATGCTGAGCAG-3’ for mouse VCAM-1-R; 5’-CCATCACCCTGTATTGCTTC-3’ for mouse ICAM-1-F and 5’-GCTGGCCGCTCAGTATCTC-3’ for mouse ICAM-1-R; 5’-CACCTAAGGCAGCATCTGC-3’ for mouse KLF2-F and 5’-TCGGTATGGCCGGAAGTC-3’ for mouse KLF2-R; 5’-TACCCCCAATGTGTCCGTC-3’ for mouse GAPDH-F and 5’-GTCCTCAGTGTAGCCCAA-3’ for mouse GAPDH-R; 5’-AAGACCTACCAAGAGGT-3’ for human KLF2-F and 5’-ACAGATGCGACTGGAATG-3’ for human KLF2-R; 5’-ACGGATTTGGTCTATGGGG-3’ for human GAPDH-F and 5’-CGCTCCTGAAAGATGGTAT-3’ for human GAPDH-R.

**Western blot analysis**

HUVEC were harvested and lysed in ice-cold 1× lysis buffer (#9803, Cell Signaling Technology, Beverly, MA) supplemented with protease inhibitor cocktail (#P8340, Sigma-Aldrich, St. Louis, MO). Protein concentrations were determined by Bradford protein assay (#500-0006, Bio-Rad, Hercules, CA) using a spectrophotometer (DU-640B, Beckman Instruments Inc., Fullerton, CA). Cell lysates were subjected to SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were then transferred onto nitrocellulose membranes (Pall, East Hills, NY), and were subsequently blocked in 1× blocking buffer (#MB-070, Rockland Immunochemicals, Inc., Gilbertsville, PA) for 1 h. Then the blots were incubated overnight at 4°C with appropriate primary antibodies (1:1000 dilution except for anti-tubulin at 1:10,000). Then after being washed 3 times with 0.1% Tween-20, membranes were incubated with Alexa Fluor 568 or 680-conjugated secondary antibodies (1:10,000 dilution; Invitrogen, Eugene, OR). Images were acquired by using an Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE). Densitometric analysis of membranes was performed using Image J software (version 1.36b, National Institutes of Health).

**Chromatin immunoprecipitation (ChIP) assay**

ChIP assays were performed as previously described. In brief, HUVEC cultured in 100-mm dishes were exposed to s- or d-flow from 0 to 24 h. Then cells were treated with 1% formaldehyde for 10 min at room temperature to cross-link the DNA-protein complex. Glycine (1.375 M, 1 ml) was added to quench formaldehyde and to terminate cross-linking reaction. Next, cells were washed twice with cold PBS and harvested by scraping using 10 ml PBS. After centrifugation at 500 g for 10 min at 4°C, cell pellets were resuspended in 10 ml lysis buffer (5 mM PIPES, 85 mM KCl, and 0.5% NP-40) and incubated on ice for 10 min. Nuclear lysates were obtained by centrifugation at 200 g for another 10 min and resuspended in nuclei lysis buffer (50 mM Tris-Cl, 10 mM EDTA, 1% SDS, and protease inhibitors). Nuclear lysates were sonicated on ice to shear DNA into fragments ranging 300–800 bp in size. Lysates were then centrifuged at 20,000 g for 15 min at 4°C to pellet the precipitated SDS, and pre-cleared by incubation
with 50 μl Protein A/G PLUS-Agarose beads (#SC-2003, Santa Cruz, Santa Cruz biotechnology, Santa Cruz, CA) at 4°C for 2 h. Then equal amounts of chromatin were incubated overnight with 1 μg rabbit anti-TXNIP or control rabbit IgG at 4°C. The protein A/G agarose beads (50 μl) were then added into the samples and incubated for 2 h at 4°C, followed by washing with high-salt wash buffer (50 mM HEPES, 500 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, and 0.1% deoxycholate) 4 times and twice with TE buffer (1 mM EDTA, and 10 mM Tris-Cl). Proteins were then digested with 1 μl proteinase K (20 μg/μl) for 2 h at 55°C and the cross-linking was reversed by incubation of the samples overnight at 65°C. DNA samples were then purified with a PCR purification kit (QIAquick PCR Purification Kit, Qiagen, Valencia, CA). Finally, PCR or real-time PCR was performed by using a pair of primers (forward: 5'-TGTCAGCGCAAGGCCCAGGCGCC-3' and reverse: 5'-CCGCCCAAGCCTTAGGCGCG-3') encompassing the consensus sequence of the KLF2 -157 bp promoter. Chromatin samples that were kept before the immunoprecipitation were used as input control.

Supplemental References
Online Figure I. **TXNIP expression is increased at branch points.** Shown is confocal immunofluorescence analysis of TXNIP expression at the branch point of the innominate artery from the aortic arch. Arrowhead points to the branch point. Region ‘G’ indicates the greater curvature. EC morphology is shown by VE-Cadherin staining.
Online Figure II. D-flow significantly increases nuclear TXNIP accumulation. HUVEC were exposed to d-flow or kept at static condition for 24 h. Total cell lysates (A), nuclear (C) and cytosolic (E) fractions were then harvested for Western blot. (B, D, F) TXNIP expression levels in each fraction were analyzed by densitometric quantifications. Results are expressed as mean ± SD of 3 independent experiments. *P<0.05.
Online Figure III. Total and membrane VCAM-1 expression is increased in d-flow area. (A) Expression of VCAM-1 was analyzed by confocal immunofluorescence. Co-localization of red (anti-VCAM-1) and green (anti-VE-Cadherin) signals in merged images indicates the membrane VCAM-1 expression. (B and C) Quantifications were performed by randomly selecting 4 lines throughout the s-flow and d-flow area (as indicated by dashed lines in the merged images in A). (B) Relative color intensity through these lines was obtained by Image-Pro and was plotted. Yellow area indicates the overlapped region from both channels which is considered to be the membrane VCAM-1 expression. (C) Total and membrane VCAM-1 expression was then quantified by calculating the area under curve (AUC). *P<0.05.
Online Figure IV. TXNIP expression is reduced in mouse aorta from EC-TXNIP KO mice. Shown is Western blot analysis of aortic TXNIP expression from EC-TXNIP KO and control littermates, respectively.
Online Figure V. Inhibition of EC-monocyte adhesion by s-flow is reversed by TXNIP overexpression. HUVEC were transfected with either TXNIP-GFP or control plasmid, kept at static condition or exposed to s-flow (12 dyn/cm²) for 24 h, and then co-incubated with THP-1 monocytes. Shown are adherent monocytes labeled by BCECF-AM. EC morphology is shown by phalloidin staining. (B) Quantification data showing the percentage of EC associated with monocytes in the presence or absence of TXNIP overexpression, respectively. Results are expressed as mean ± SD of 3 independent experiments.
Online Figure VI. No significant differences in blood pressure and heart rate between control and EC-TXNIP knockout mice. Shown are systolic blood pressure (A) and heart rate (B) from EC-TXNIP knockout mice and control littermates. Data are expressed as mean ± SD from 5 mice in each group.
Online Figure VII. No differences in PBMC TXNIP expression between EC-TXNIP KO mice and control littermates. Mouse PBMC were freshly isolated by density-gradient centrifugation. Shown is Western blot analysis of PBMC TXNIP expression from control and EC-TXNIP KO mice.
Online Figure VIII. NF-κB activation in response to d-flow is reversed by TXNIP depletion. HUVEC were pretreated with scramble control or TXNIP-specific siRNA for 48 h, transfected with NF-κB luciferase reporter, and exposed to d-flow or kept at static condition for an additional 24 h. Cell were harvested and the luciferase activities were measured. Results are expressed as mean ± SD of 3 independent experiments. *P<0.05.
Online Figure IX. Expression of eNOS is repressed by TXNIP. (A) HUVEC in 100-mm dish were transfected with 3 μg TXNIP-GFP or control plasmid, and then exposed to s-flow or kept at static condition for 24 h. (B) HUVEC were exposed to d-flow for the indicated times. eNOS expression levels were analyzed by Western blot.
Online Figure X. A scheme describing the TXNIP-mediated cross talk between s-flow (anti-inflammatory) and d-flow (pro-inflammatory) pathways.