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Thioredoxin-Interacting Protein Mediates Sustained VEGFR2 Signaling in Endothelial Cells required for Angiogenesis

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Abstract

Objective—Thioredoxin-interacting protein (TXNIP) is an α -arrestin protein whose function is important for the regulation of vascular endothelial growth factor receptor 2 (VEGFR2) signaling and endothelial cell (EC) survival. Since VEGFR2 is critical for angiogenesis, we explored the role of TXNIP in VEGF-induced angiogenesis.

Approach/Results—TXNIP knockdown inhibited VEGF-induced EC tube formation and proliferation in cultured human umbilical vein EC (HUVEC). To elucidate the mechanism by which TXNIP altered VEGFR2 signaling in HUVEC, we studied phosphorylation of VEGFR2, PLC γ 1, eNOS, and Akt. TXNIP knockdown significantly decreased phosphorylation of VEGFR2 and PLC γ 1 at times > 5min, but phosphorylation was unchanged at 2min; as was Akt and eNOS phosphorylation. Cell surface biotinylation assay showed that TXNIP knockdown significantly attenuated VEGFR2 internalization. These results suggested that TXNIP was required for sustained VEGFR2 signaling, which is mediated largely by internalized VEGFR2. Rab5 knockdown to inhibit the trafficking and fusion of early endosomes significantly blocked VEGF-induced VEGFR2 internalization and phosphorylation of VEGFR2 and PLC γ 1. Immunofluorescence and co-immunoprecipitation showed that TXNIP was part of a complex that included Rab5 and VEGFR2. Finally, TXNIP knockdown prevented the association of VEGFR2 and Rab5.

Conclusions—Our results show that TXNIP is essential for VEGFR2 internalization in Rab5 positive endosomes, which is required for EC growth and angiogenesis.

Keywords

VEGFR2; Thioredoxin-interacting protein; Rab5; Angiogenesis; Signaling

Introduction

Angiogenesis is the formation of new blood vessels from the existing vascular network that includes endothelial cell (EC) proliferation, tube formation, and migration, involving several growth factors^{1, 2}. Among them, vascular endothelial growth factor (VEGF) is a major

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Disclosures

None.

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proangiogenic factor that regulates EC function by binding to the VEGF receptor 2 (VEGFR2)^{3,4}. In particular, the phosphorylation of VEGFR2 is a key event for internalization of the activated receptor and signaling⁵. Regulation of internalization and localization of VEGFR2 promotes numerous downstream signal transduction pathways that lead to proliferation, migration, permeability, and differentiation of vascular EC^{6,7}. Therefore, internalization of VEGFR2 is considered an important mechanism through which cells regulate signal transduction and EC functions. Until recently, most studies on VEGFR2 internalization have focused on receptor ubiquitylation with degradation⁸⁻¹⁰. However, the mechanisms by which internalization of VEGFR2 mediates angiogenesis are not fully understood.

Thioredoxin-interacting protein (TXNIP) is an α -arrestin family member and acts as a scaffold protein with several protein-protein interacting domains^{11,12}. TXNIP regulates inflammation in EC by binding to and inhibiting thioredoxin (TRX) in a redox-dependent fashion^{13,14}. We previously showed that the TXNIP-TRX1 complex translocated to the plasma membrane in response to physiological concentrations of hydrogen peroxide (H₂O₂) or tumor necrosis factor (TNF)¹⁵. Membrane TXNIP plays an essential role in VEGFR2 phosphorylation, EC survival, and EC migration. However, the mechanisms by which TXNIP regulates downstream VEGFR2 signaling and angiogenesis have not been elucidated.

In the present study, we found that TXNIP was required for VEGF mediated angiogenesis as shown by decreased EC tube formation and proliferation. TXNIP was also required for VEGF signaling via regulation of VEGFR2 internalization in Rab5 positive endosomes.

Results

TXNIP is required for VEGF-induced Tube formation and Proliferation in EC

To investigate the role of TXNIP in angiogenesis, we analyzed EC tube formation in vitro. In control siRNA treated cells, VEGF treatment increased tube length by 5.4 ± 1.1 fold (Figure 1A, 1B and 1E) and tube numbers by 5.8 ± 1.5 fold (Figure 1F) compared to PBS treated cells. Tube length and tube numbers were significantly reduced by TXNIP knockdown (1.3 ± 0.6 fold and 2.2 ± 0.8 fold, respectively; Figure 1C-F, $P < 0.05$). As EC proliferation is a key step for angiogenesis, we evaluated the role of TXNIP on VEGF-induced proliferation in HUVEC. As shown in Figure 1G, VEGF increased the number of cells (1.6 ± 0.2 -fold), which was significantly decreased by TXNIP knockdown (1.2 ± 0.3 -fold, $P < 0.05$). To confirm a role for TXNIP in cell proliferation, we performed a BrdU incorporation assay. In control siRNA treated cells, VEGF treatment increased cell proliferation by 3.9 ± 0.8 fold compared to PBS treated cells, which was significantly reduced by TXNIP knockdown (1.5 ± 0.3 -fold, $P < 0.05$, Figure 1H). These data suggest that TXNIP is required for VEGF-induced EC tube formation and proliferation.

TXNIP defines two VEGF-VEGFR2 signal pathways that differ temporally

To investigate the functional role of TXNIP in VEGF-mediated signaling events in EC, we measured phosphorylation of VEGFR2 and PLC γ -1 using HUVEC transfected with TXNIP siRNA or control siRNA. TXNIP siRNA efficiently reduced endogenous TXNIP expression, compared to control siRNA (Figure 2A). In control siRNA treated cells VEGF stimulated phosphorylation of VEGFR2 (Figure 2A and 2B) and PLC γ 1 (Figure 2A and 2C) as early as 2 min and the phosphorylation was maintained for 15 min. Interestingly, TXNIP knockdown did not alter the phosphorylation of VEGFR2 and PLC γ 1 at 2 min, but significantly attenuated phosphorylation from 5 to 15 min of VEGF stimulation. Another rapidly activated VEGF pathway is phosphorylation of Akt-eNOS. To investigate the role of

TXNIP in this pathway, HUVEC were transfected with control siRNA, or TXNIP siRNA. Upon VEGF stimulation, both Akt and eNOS were rapidly phosphorylated at 1 and 2 min with no significant difference after TXNIP knockdown (Figure IA in the online-only Data Supplement). These data show that TXNIP is not required for VEGFR2 regulation of eNOS and Akt, defining a novel role for TXNIP in EC.

Protein tyrosine phosphatases do not mediate TXNIP dependent VEGFR2 phosphorylation

Protein tyrosine phosphatases (PTPs) such as PTP1B and Src homology 2 (SH2) domain-containing protein tyrosine phosphatase (SHP)-1 inhibit VEGFR2 phosphorylation and EC functions^{16, 17}. Therefore, we hypothesized that TXNIP regulates VEGFR2 phosphorylation through inhibiting PTP1B or SHP1. Specific inhibition of PTP1B or SHP1 by drugs or siRNA knockdown increased VEGF-stimulated VEGFR2 phosphorylation. However, the TXNIP effect on VEGFR2 phosphorylation was not altered by PTP1B knockdown (Figure IIA in the online-only Data Supplement) or SHP1 inhibition (Figure IIB in the online-only Data Supplement). These data suggest that inhibiting of PTP1B and SHP1 by TXNIP is not the mechanism of TXNIP-mediated regulation of VEGFR2 phosphorylation.

TXNIP is required for VEGF-induced VEGFR2 internalization

We hypothesized that TXNIP may regulate VEGFR2 internalization for two reasons. First, receptor internalization is directly linked to VEGFR2 phosphorylation and downstream signaling⁷. Second, TXNIP depletion did not affect VEGFR2 phosphorylation at 2 min, while sustained VEGFR2 signaling was inhibited. To study VEGFR2 internalization, cell-surface VEGFR2 in HUVEC was labeled with a membrane-impermeable biotin derivative and the cells were then exposed to VEGF at 37 °C to allow endocytosis. As controls, cells were also biotin labeled and stimulated with VEGF for 15 min at 4 °C to prevent VEGFR2 internalization. After VEGF exposure, the remaining cell-surface biotin was cleaved with glutathione, and internalized proteins were collected using streptavidin-conjugated beads. The amount of internalized VEGFR2 was determined by western blotting. There was a significant increase of internalized VEGFR2 in control siRNA treated cells exposed to VEGF (16.4±4.1 and 27.2±5.9-fold at 5 and 15 min, Figure 3A and 3B). In contrast, internalized VEGFR2 was decreased in TXNIP knockdown cells following VEGF stimulation (6.1±2.1 and 7.1±3.3-fold at 5 and 15 min, Figure 3A and 3B). These results indicate that TXNIP regulates VEGFR2 internalization.

Rab5 is involved in TXNIP-mediated VEGFR2 internalization and signaling

After VEGF stimulation, VEGFR2 internalization involves a Rab5 GTPase dependent endocytotic pathway. Furthermore, endocytosis of activated VEGFR2 is required for multiple signal pathway in EC^{5, 18, 19}. Rab5 is necessary for the early steps of endocytosis such as budding and docking/fusion activities. Manipulation of Rab5 function leads to early endosome-specific modulation of VEGFR2 signaling²⁰⁻²². Thus, we determined whether VEGFR2 internalization in endosomes is critical for the sustained VEGFR2 phosphorylation using Rab5 siRNA to specifically block the internalization system. We found that Rab5 siRNA effectively blocked VEGF-stimulated VEGFR2 internalization (Figure 4A and 4B). Importantly, we demonstrated that blocking VEGFR2 internalization by Rab5 siRNA attenuated sustained phosphorylation of VEGFR2 and PLCγ1 but did not affect the early 2 min phosphorylation (Figure 4C-E). In addition, Rab5 depletion did not affect phosphorylation of Akt and eNOS in response to VEGF (Fig. IB in the online-only data supplement), indicating the endosome independent of VEGF-stimulated phosphorylation of Akt and eNOS. To prove VEGFR2 internalization is functionally required for angiogenesis, we examined tube formation. As shown in Figure 4F and 4G, VEGF-induced tube length was significantly reduced by Rab5 knockdown compared to control siRNA (1.3±0.5-fold

versus 4.5 ± 1.2 -fold, $P < 0.05$), indicating the requirement for VEGFR2 internalization to induce angiogenesis.

We next determined whether TXNIP localizes to Rab5 positive endosomes. First, we performed immunofluorescence on cultured HUVEC to show the co-localization of TXNIP and Rab5 in response to VEGF. Colocalization of TXNIP and Rab5 at membrane and cytosolic region significantly increased after 5min of VEGF stimulation (Figure 5A and Figure III in the online-only Data Supplement). The peak in colocalization of TXNIP and Rab5 occurred at 15 min (8.2 ± 2.0 -fold, $P < 0.05$, Figure 5A). To confirm that TXNIP interacts with early endosomes we used the early endosomal marker 1 (EEA1), a well-known Rab5 effector that functions as a tethering protein in early endosome fusion²³. In response to VEGF, TXNIP colocalized with EEA1 at membrane and cytosol (peak at 5 –15 min, Figure IV in the online-only Data Supplement). In addition, we showed that TXNIP and VEGFR2 traffic together after 5 min of VEGF treatment at membrane and cytosol, but very little at 2min and 30min (Figure V in the online-only Data Supplement). Consistent with these results, co-immunoprecipitation studies showed that VEGF stimulates the association of TXNIP with Rab5 and VEGFR2 complex after 5 min but not at 2 min (Figure 5B). This suggests that TXNIP and VEGFR2 translocate together to endosomes after 5 min of VEGF stimulation (Figure 5B). Further, TXNIP siRNA blocked the association of VEGFR2 with Rab5 in response to VEGF (1.3 ± 0.3 and 1.1 ± 0.2 -fold at 5 and 15 min) compared with control siRNA (7.8 ± 1.9 and 8.2 ± 2.0 -fold at 5 and 15 min, Figure 5C), indicating a critical role of TXNIP in the Rab5 and VEGFR2 complex formation. In summary, the formation of VEGFR2 and Rab5 complex mediated via TXNIP is critical for VEGFR2 internalization and sustained VEGFR2 signaling.

Discussion

The major findings of this study are that TXNIP is essential for VEGFR2 internalization in Rab5 positive endosomes, which is required for EC growth and angiogenesis. Based on our data, we propose the following model for the role of TXNIP in VEGF-VEGFR2 signaling in EC (Figure 6). The binding of VEGF induces autophosphorylation of Tyr1175 in the cytoplasmic domain of VEGFR2 receptor, which is required for VEGFR2 activation and angiogenesis²⁴. The earliest signaling events that occur within 2 min such as phosphorylation of VEGFR2, PLC γ , Akt and eNOS (Figure 2), do not require TXNIP (Figure IA in the online-only Data Supplement). In contrast, sustained signaling such as persistent VEGFR2 and PLC γ 1 phosphorylation requires TXNIP. A key finding was that TXNIP-mediated sustained signaling occurred in internalized VEGFR2 localized to Rab5 positive endocytotic vesicles (Figure 4 and Figure 5). TXNIP dependent signaling in EC was found to have significant physiologic effects as shown by findings that TXNIP was required for VEGF-mediated tube formation and proliferation.

TXNIP is a member of the α -arrestin family that functions as an intracellular scaffold, which participates in cellular signaling by formation of signaling complexes and localization of signaling components in the cell^{11, 12}. VEGF-VEGFR2 signaling in EC appears to be dependent on TXNIP as shown in the present study. We showed previously that TXNIP was required for VEGFR2 activation and EC survival in response to low concentrations of H₂O₂ and TNF- α ¹⁵. These data support our concept that TXNIP plays a critical role in regulating VEGFR2 signaling and angiogenesis in EC.

A novel finding of the present study is that TXNIP appears to be required for the earliest stage of VEGFR2 internalization. Receptor tyrosine kinases are regulated by endocytosis through the internalization of plasma membrane receptors²⁵⁻²⁷. For example, the internalization of epidermal growth factor receptor (EGFR) is mediated by clathrin-mediated

endocytosis and is essential for sustained EGFR signaling²⁸. Internalized receptor is delivered from the plasma membrane to early endosomes in endocytic vesicles²⁹ and canonically defined Rab5 is an early endosome marker³⁰. We showed that VEGFR2 internalization into endosomes is critical for sustained VEGFR2 signaling and angiogenesis using Rab5 siRNA to specifically block the internalization system. Furthermore, we demonstrated that blocking VEGFR2 internalization by Rab5 siRNA attenuated sustained phosphorylation of VEGFR2 and PLC γ 1 but did not affect the early 2 min phosphorylation (Figure 4A and 4B). These data demonstrate that VEGFR2 internalization is critical for sustained VEGFR2 signaling.

Our findings have potential clinical implications. We identified TXNIP as a critical component in sustained VEGFR2 signaling, such as VEGF-induced tube formation, proliferation and angiogenesis. Inhibiting VEGFR2 signaling with antibodies and small molecules has demonstrated efficacy in diseases such as cancer and proliferative retinopathy by limiting angiogenesis³¹⁻³³. However, these therapies are associated with side effects such as hypertension due to inhibiting VEGFR2 signaling that includes generation of nitric oxide. The present findings suggest that the TXNIP-dependent VEGFR2 signal pathway is separate from the VEGFR2 pathway that activates eNOS. If this is true in vivo, inhibiting TXNIP function in EC specifically might limit side effects dependent on nitric oxide. Also, because TXNIP is an α -arrested and depleting TXNIP mimics the effect of inhibiting Rab5 activity, it is likely that other receptor tyrosine kinases will be regulated by TXNIP. Future studies will be required to elucidate the specific domains of TXNIP and interacting proteins that are responsible for sustained VEGFR2 signaling required for angiogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Significance

Regulation of receptor internalization is considered as an important mechanism that regulates downstream cell signaling pathways and cell functions. In particular, Internalization of vascular endothelial growth factor receptor 2 (VEGFR2) is essential for VEGF downstream signaling that lead to endothelial cell (EC) proliferation, migration and survival. However, the mechanisms by which internalization of VEGFR2 contribute to VEGFR2 signaling and EC angiogenesis are not clear. The novel findings of this study are that Thioredoxin-interacting protein (TXNIP) is required for sustained VEGFR2 activation and EC proliferation and tube formation. Specifically, TXNIP regulates VEGF signaling via regulation of VEGFR2 internalization in Rab5 endocytotic vesicles. These findings provide new insight into the role of TXNIP as a critical regulator for EC angiogenesis by regulating endosome-dependent VEGFR2 signaling and VEGFR2 internalization.

Figure 1. Effect of TXNIP on VEGF-induced tube formation and proliferation in ECs
 (A-D) HUVEC were transfected with control siRNA or TXNIP siRNA. After 48 hrs, the cells were seeded on 96-well plates at 5×10^4 cells/well coated with growth factor-reduced Matrigel and maintained in 1% FBS growth media contains VEGF (20ng/ml) for 18 hrs at 37 °C. Scale Bar, 100 μ m. (E-F) Quantification of tube length (E) and the number of tubes per field (F) were analyzed with the use of Image J and Image pro, respectively. (G-H) HUVEC were transfected with control siRNA or TXNIP siRNA. After 48 hrs, the cells were treated with VEGF (20 ng/ml) or PBS in 0.5% FBS medium for 48 hrs. The number of cells was counted after trypsinization (G) and DNA synthesis was measured by BrdU incorporation (H) * $P < 0.05$ vs. control siRNA +VEGF (mean $\pm$ SD; n=3).

Figure 2. TXNIP is required for activation of VEGFR2 and PLCγ1 in EC

(A) HUVEC were transfected with control siRNA or TXNIP siRNA. After 48 hrs, the cells were treated with VEGF (10 ng/ml) for the indicated times. Cell lysates were immunoblotted with p-VEGFR2 (Y1175), VEGFR2, p-PLCγ1 (Y783), and PLCγ1. (B-C) Lower panels are quantification analysis. * $P < 0.05$ vs. control siRNA+VEGF (mean $\pm$ SD; n=3).

Figure 3. TXNIP regulates VEGFR2 internalization

(A) HUVEC were transfected with control siRNA or TXNIP siRNA for 48 hrs and the cells were surface labeled with biotin and stimulated with VEGF (10 ng/ml) for the indicated times. After stripping remaining cell-surface biotin, cell lysates were immunoprecipitated with streptavidin beads to bind the internalized biotinylated proteins. Samples were immunoblotted for VEGFR2 to determine internalized VEGFR2 (upper panel). Total cell lysates were also immunoblotted with VEGFR2 antibodies to detect total (surface + internalized) VEGFR2 levels (lower panel). (B) Internalized VEGFR2 was quantified relative to the total amount of VEGFR2 using Image J. * $P < 0.05$ vs. control siRNA + VEGF (mean $\pm$ SD; $n=3$).

Figure 4. Rab5 is involved in VEGF-induced VEGFR2 internalization and signaling

(A) HUVEC were transfected with control siRNA or Rab5 siRNA for 48 hrs and the cells were surface labeled with biotin and stimulated with VEGF (10 ng/ml) for indicated times. After stripping remaining cell-surface biotin, cell lysates were immunoprecipitated with streptavidin beads to bind the internalized biotinylated proteins. Samples were immunoblotted for VEGFR2 to determine internalized VEGFR2 (upper panel). Total cell lysates were also immunoblotted with VEGFR2 antibodies to detect total (surface + internalized) VEGFR2 levels (lower panel). (B) Internalized VEGFR2 was quantified relative to the total amount of VEGFR2 using Image J. * $P < 0.05$ vs. control siRNA + VEGF (mean $\pm$ SD; $n = 3$). (C) HUVEC were transfected with control siRNA or Rab5 siRNA. After

48 hrs, the cells were treated with VEGF (10 ng/ml) for indicated times. Cell lysates were immunoblotted with p-VEGFR2 (Y1175), VEGFR2, p-PLC γ 1 (Y783), and PLC γ 1. (D-E) Quantification analysis. * $P < 0.05$ vs. control siRNA+VEGF (mean $\pm$ SD; n=3). (F) HUVEC were transfected with control siRNA or Rab5 siRNA. After 48 hrs, the cells were seeded on 96-well plates at 5×10^4 cells/well coated with growth factor-reduced Matrigel and maintained in 1% FBS growth media contains VEGF (20ng/ml) for 18 hrs at 37 °C. Scale Bar, 100 μ m. (G) Quantification of tube length was analyzed with the use of Image J. * $P < 0.05$ vs. control siRNA +VEGF (mean $\pm$ SD; n=3).

Figure 5. TXNIP is critical for Rab5 and VEGFR2 complex in response to VEGF
 HUVEC were untreated or treated with 10 ng/ml VEGF for indicated time (A and B). (A) Quantification of TXNIP and Rab5 binding (punctae spots) were performed by image-Pro. * $P < 0.05$ vs. unstimulated control (mean $\pm$ SD; $n = 6$). (B) Immunoblot analysis of anti-TXNIP immunoprecipitates from the cell lysates with anti-VEGFR2 or anti-Rab5. Immunoblot analysis of total cell lysates from IP samples was performed with anti-VEGFR2, TXNIP or Rab5. (C) HUVEC were transfected with control siRNA or TXNIP siRNA. After 48 hrs, the cells were treated with VEGF (10 ng/ml) for indicated times. Cell lysates were used to Immunoprecipitate Rab5 and immunoblotting for VEGFR2. Immunoblot analysis of total cell lysates from IP samples was performed with anti-VEGFR2, TXNIP or Rab5.

Figure 6. Model for TXNIP-mediated VEGFR2 signaling in EC angiogenesis

VEGF triggers autophosphorylation of VEGFR2 (Y1175), which is internalized inside endocytotic vesicles and leads to PLCγ1 phosphorylation in TXNIP-dependent. TXNIP sustains VEGFR2 signaling that required for EC tube formation and proliferation in angiogenesis. On the other hand, transient VEGFR2 signaling (p-AKT/p-eNOS pathway) is independent of TXNIP.