Published in final edited form as:

Oncogene. 2011 May 5; 30(18): 2098–2107. doi:10.1038/onc.2010.590.

The fungicide ciclopirox inhibits lymphatic endothelial cell tube formation by suppressing VEGFR-3-mediated ERK signaling pathway

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Abstract

Ciclopirox olamine (CPX), an off-patent antifungal agent used to treat mycoses of skin and nails, has recently been demonstrated to be a potential anticancer agent. However, the underlying mechanism is not well understood. Here for the first time we show that CPX inhibited lymphangiogenesis in an *in vitro* model (tube formation). This effect was in part associated with inhibition of vascular endothelial growth factor receptor 3 (VEGFR-3) expression, as overexpression of VEGFR-3 conferred partial resistance to CPX inhibitory effect on tube formation in lymphatic endothelial cells (LECs), whereas downregulation of VEGFR-3 mimicked the effect of CPX, blocking the tube formation. Further study revealed that CPX did not alter mRNA level, but inhibited protein synthesis and promoted protein degradation of VEGFR-3. In addition, we found that CPX inhibited phosphorylation of the extracellular signal-related kinase 1/2 (ERK1/2), a downstream effector of VEGFR-3. Overexpression of VEGFR-3 attenuated CPX inhibition of ERK1/2 phosphorylation, whereas downregulation of VEGFR-3 inhibited extracellular signal-related kinase 1/2 (ERK1/2) phosphorylation in LECs. Ectopic expression of constitutively active mitogen -activated protein kinase kinase 1 (MKK1) resulted in activation of

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ERK1/2, and partially prevented CPX inhibition of LEC tube formation. The results suggest that CPX inhibits LEC tube formation at least in part through inhibiting VEGFR-3-mediated ERK signaling pathway.

Keywords

Ciclopirox; lymphatic endothelial cells; tube formation; VEGFR-3; ERK

Introduction

Ciclopirox olamine (CPX) ,an off-patent synthetic antifungal agent, has been used topically to treat fungal and yeast infection of skin or mucosa for more than 20 years (Dittmar et al., 1973; Jue et al., 1985). Recent studies have shown that CPX induced cell death in primary human acute myeloid leukemia (AML) cells and inhibited engraftment of primary AML cells in NOD/SCID mouse models without gross organ toxicity or loss of body weight (Eberhard et al., 2009). Most recently we have further demonstrated that CPX displayed anticancer activity *in vitro* and *in vivo* by inhibiting proliferation and inducing apoptosis of solid tumor cells, such as human rhabdomyosarcoma (Rh30), breast carcinoma (MDA-MB231), and colon adenocarcinoma (HT-29) cells (Zhou et al., 2010). CPX inhibition of cell proliferation was associated with downregulation of cyclins (A, B1, D1 and E) and cyclin-dependent kinases (CDK2 and CDK4), and upregulation of CDK inhibitor $p21^{Cip1}$, resulting in hypophosphorylation of retinoblastoma protein and consequently slowing down cell cycle progression from G_1/G_0 to S phase (Zhou et al., 2010). CPX induction of apoptosis was mainly related to downregulation of anti-apoptotic proteins (Bcl-xL and survivin) and increased cleavage of Bcl-2. These findings suggest that CPX is a potential antitumor agent.

Lymphangiogenesis, like angiogenesis, plays an important role in promoting tumor growth and metastasis (Mandriota et al., 2001; Skobe et al., 2001; Stacker et al., 2001; Karpanen and Alitalo, 2008).Targeting lymphatic system is a promising strategy for treatment or prevention of tumor growth and metastasis (Pepper, 2001; Cueni and Detmar, 2006). Studies have shown that CPX inhibits angiogenesis in human endothelial cells (Clement et al., 2002), although this remains controversial (Linden et al., 2003). To our knowledge, the effect of CPX on lymphangiogenesis has not been reported, which prompted us to study whether CPX possesses anti-lymphangiogenic activity.

Vascular endothelial growth factor receptor 3 (VEGFR-3), also known as fms-like tyrosine kinase 4 (Flt-4), is primarily expressing on the surface of lymphatic endothelial cells (LECs) (Kaipainen et al., 1995). Ligand (VEGF-C/D) binding activates VEGFR-3, leading to activation of the downstream signaling molecules, such as phosphatidylinositol 3-kinase (PI3K)/Akt and mitogen-activated protein kinases (MAPKs) (Karpanen and Alitalo, 2008; Mandriota et al., 2001; Skobe et al., 2001; Stacker et al., 2001; Wissmann and Detmar, 2006). VEGFR-3 signaling plays a critical role in LEC survival and lymphangiogenesis (8), as well as metastasis (Mandriota et al., 2001; Skobe et al., 2001; Stacker et al., 2001). Thus, targeting VEGFR-3 pathway is a potential strategy for cancer prevention and treatment.

The study of lymphangiogenesis has been impeded by the difficulties in the isolation and propagation of LECs from different organs (Makinen et al., 2001; Nisato et al., 2004). To overcome the above limitations, we selected a 'conditionally immortalized' line of murine LECs, which express SV40 large T and retain their "lymphatic" endothelial characteristics after repeated passages (Ando et al., 2005). LECs *in vitro* culture still remain the ability to sprout, elongate, migrate, and reorganize to form a tube structure within 2-3 h, a process called tube formation, and this reflects key aspects and represents a crucial process of lymphangiogenesis (Fang et al., 2009). We thus used this LEC tube formation model, a simple and reproducible approach, to study the effect of CPX on lymphangiogenesis *in vitro*. Our results reveal that CPX inhibited LEC tube formation, which was at least attributed to inhibition of protein expression of VEGFR-3 and phosphorylation of ERK1/2.

Results

CPX inhibits LEC tube formation

Studies have shown that CPX inhibits angiogenesis (Clement et al., 2002), although this is controversial (Linden et al., 2003). To further unveil whether CPX inhibits lymphangiogenesis, we chose murine LEC tube formation as an *in vitro* model for lymphangiogenesis. Treatment with CPX (0-5 μM) for 24 h did not obviously influence LEC cell viability according to cell morphology (Fig.1A, Bottom panel), but resulted in a concentration-dependent inhibition of LEC tube formation (Fig.1A, Upper panel). At 2.5 and 5 μM, CPX inhibited the tube formation by approximately 70% and 90%, respectively, comparing with the control group (Fig. 1C). Furthermore, CPX (5 μM) inhibited LEC tube formation in a time-dependent manner as well (Fig. 1B, Bottom panel), despite no apparent effect on cell viability (Fig.1B, Upper panel). After treatment for 4 h, CPX (5 μM) was able to significantly block the tube formation (by ~20%). When LECs were treated with CPX (5 μM) for 24 h, the tube formation was inhibited by ~90%, comparing with the control group (Fig. 1D).

CPX inhibition of LEC tube formation is associated with suppressing VEGFR-3 protein expression

Since VEGFR-3 is primarily expressed in LECs (13), and essential for lymphangiogenesis (Karpanen and Alitalo, 2008; Mandriota et al., 2001; Skobe et al., 2001; Stacker et al., 2001), we investigated whether CPX inhibits LEC tube formation by targeting VEGFR-3. When LECs were exposed to CPX for 24 h, a pronounced reduction of protein expression of VEGFR-3 was detected at 1-5 μM by Western blot analysis (Fig.2A). When the cells were treated with CPX at 5 μM, a time-dependent inhibition of VEGFR-3 expression was also observed. Treatment with CPX for 8 h markedly reduced VEGFR-3 level. Prolonged treatment with CPX resulted in more reduction of VEGFR-3 (Fig.2B).

To determine the role of VEGFR-3 in CPX inhibition of LEC tube formation, LEC cells (LEC/VEGFR-3) stably overexpressing VEGFR-3 were generated by transfection with p3×Flag-VEGFR-3-TV1 plasmid. In comparison with the control cells (LEC/V) transfected with the empty vector, approximately 3-fold increase of VEGFR-3 protein expression was detected in LEC/VEGFR-3 cells (Fig. 2C). Treatment with CPX (5 μM) for 24 h reduced

protein expression of VEGFR-3 by ~90% (Fig. 2C), and consequently inhibited the tube formation by ~90% in LEC/V cells (Fig.2D). When LEC/VEGFR-3 cells were treated with CPX (5 μM) for 24 h, VEGFR-3 protein expression was downregulated by ~50%, but the VEGFR-3 protein level was still slightly higher than the basal level in the control (LEC/V) cells (Fig. 2C). Of interest, overexpression of VEGFR-3 significantly increased the tube formation and conferred high resistance to CPX inhibition of the tube formation (Fig. 2D), suggesting that CPX inhibits LEC tube formation in part by suppressing VEGFR-3 protein expression.

To further confirm the role of VEGFR-3 in CPX inhibition of LEC tube formation, RNA interference technology was applied. Infection with lentiviral shRNA to VEGFR-3 silenced the protein expression of VEGFR-3 by ~90%, comparing with the controls infected with lentiviral shRNA to GFP (control) (Fig.3A). Downregulation of VEGFR-3 mimicked the effect of CPX, inhibiting the tube formation by ~90%. Under this condition, no synergistic or additive inhibitory effect on the tube formation was observed by treatment with CPX (Fig.3B). However, when LECs were infected with half amount of the lentiviral shRNA, and VEGFR-3 protein expression was downregulated by ~50% (Fig.3C), a synergistic inhibitory effect on the tube formation was observed in combination with 2.5 μM CPX treatment (Fig. 3D). This is evidenced by the findings that half amount of VEGR-3 shRNA or CPX (2.5 μM) alone inhibited LEC tube formation by approximately 42% and 45%, respectively, whereas a combination treatment inhibited LEC tube formation by ~65% (Fig.3D). The results suggest that CPX may have a potential to synergize the anti-lymphangiogenic effects of VEGFR-3 inhibitors.

CPX inhibits protein expression of VEGFR-3 by inhibiting protein synthesis and promoting protein degradation of VEGFR-3 in LECs

CPX inhibition of protein expression of VEGFR-3 may occur at transcriptional, translational and/or post-translational level. To this end, firstly, semi-quantitative RT-PCR was employed to determine whether CPX affects VEGFR-3 mRNA expression. As shown in Fig.4A, there was no any apparent change of the mRNA level, regardless of treatment with CPX at 0-5 μM 24 h or at 5 μM for 0-24 h. This was confirmed by quantitative real time PCR (qRT-PCR) as well (see supplemental data, Fig.1S).

Next, ^{35}S-Met/Cys labeling was used to determine whether CPX impacts VEGFR-3 protein synthesis. After LECs, pretreated with CPX at 0-5 μM 24 h or at 5 μM for 0-24 h, were pulsed with ^{35}S-Met/Cys for 4 h, autoradiography revealed that pretreatment with CPX at 2.5-5 μM for 24 h inhibited incorporation of ^{35}S-Met/Cys into VEGFR-3 by ~50-70%, and pretreatment with CPX at 5 μM for 12-24 h suppressed incorporation of ^{35}S-Met/Cys into VEGFR-3 by ~70%, comparing with the controls (Fig.4B).

Furthermore, to determine whether CPX influences protein degradation of VEGFR-3, LECs, grown in 10% FBS-DMEM medium, were exposed to cycloheximide (CHX, 50 μg/ml), an inhibitor of eukaryotic protein synthesis by preventing initiation and elongation on 80S ribosomes, in the presence or absence of CPX (5 μM), for up to 12 h, followed by Western blot analysis. It appeared that CPX treatment remarkably promoted VEGFR-3 protein turn-over rate. As illustrated in Fig.4C, considerable level of VEGFR-3 protein was still

detectable when the cells were treated with CHX alone for 8 h. However, a very low level of VEGFR-3 protein was observed when the cells were treated with CHX + CPX only for 4 h. Collectively, our data indicate that CPX did not alter mRNA level, but inhibited protein synthesis and promoted protein degradation of VEGFR-3, thereby inhibiting protein expression of VEGFR-3 in LECs.

CPX inhibits LEC tube formation by targeting VEGFR-3-mediated ERK1/2 pathway

PI3K/Akt and MAPK pathways are the two major downstreams of VEGFR-3 (Wissmann and Detmar, 2006). We hypothesized that CPX inhibits LEC tube formation through targeting these pathways. To our surprise, when LECs were treated with CPX (0-5 μM) for 24 h or 5 μM for 0-24 h, PI3K/Akt pathway was not affected. This is evidenced by the findings that CPX failed to alter protein expression or phosphorylation of Akt (Fig.5A,B). Treatment with CPX did not obviously affect cellular protein expression or phosphorylation of JNK and p38 MAPK either, but dramatically inhibited phosphorylation of ERK1/2, despite no effect on total protein levels of ERK1/2 (Fig.5A,B), suggesting a selective inhibition of the ERK pathway in the LECs.

To examine whether CPX inhibition of phosphorylation of ERK1/2 is due to inhibition of VEGFR-3 protein expression, LEC/VEGFR-3 and LEC/V cells were treated with CPX (5 μM) for 24 h. By Western blot analysis, overexpression of VEGFR-3 upregulated phosphorylation of ERK1/2, and conferred high resistance to CPX inhibition of phosphorylation of ERK1/2 (Fig.5C). In contrast, downregulation of VEGFR-3 by lentiviral shRNA mimicked the effect of CPX, reducing phosphorylation of ERK1/2 (Fig.5D). The results support the notion that CPX inhibition of ERK1/2 phosphorylation is a consequence of downregulation of VEGFR-3 protein expression.

To determine whether CPX inhibition of LEC tube formation is really attributed to inhibition of ERK1/2 pathway, we generated recombinant adenoviral vector (Ad-MKK1-R4F) expressing Flag-tagged constitutively active MKK1, which activates ERK1/2 (Mansour et al., 1994). As seen in Fig.6A, Flag-MKK1 was expressing in the LECs infected with Ad-MKK1-R4F, but not Ad-GFP (control). Furthermore, ectopic expression of constitutively active MKK1 induced a robust phosphorylation of ERK1/2 in LECs. Treatment with CPX (5 μM) for 24 h inhibited phosphorylation of ERK1/2 in both Ad-GFP infected (control) and Ad-MKK1-R4F infected cells. However, the level of phospho-ERK1/2 in Ad-MKK1-R4F infected cells treated with CPX was comparable to the basal level in the control cells (Fig.6A). Noticeably, expression of constitutively active MKK1, but not GFP, rendered high resistance to CPX inhibition of LEC tube formation (Figure.6B). As a control, U0126 (a selective inhibitor of MKK1/2, upstream of ERK1/2) was utilized. We found that U0126 (5μM) alone inhibited phospho-ERK1/2 and LEC tube formation profoundly (Fig.6C,D). In combination, CPX (5 μM) did not enhance U0126 inhibition of the tube formation (Fig.6D), although more inhibition of phospho-ERK1/2 was seen (Fig. 6C). The results suggest that CPX inhibits LEC tube formation through targeting VEGFR-3-mediated ERK pathway.

Discussion

Recent studies have shown that CPX, a fungicide used to treat mycoses of skin and nails for over two decades, also inhibited cell proliferation and induced apoptosis of cancer cells in culture and in xenografts in mice (Eberhard et al., 2009; Zhou et al., 2010). Here, for the first time, we further show that CPX inhibited murine LEC tube formation, an *in vitro* lymphangiogenesis model. This is consistent with the findings that CPX inhibited human umbilical vein endothelial cell (HUVEC) tube formation, an *in vitro* angiogenesis model (Clement et al., 2002). It is widely accepted that lymphangiogenesis, like angiogenesis, is crucial for tumor growth and metastasis (Pepper, 2001; Wissimann and Detmar, 2006). Taken together, these findings strongly suggest that CPX is a potential anticancer agent.

VEGFR-3 is a transmemberane receptor tyrosine kinase in LECs and activated by binding of the ligands, VEGF-C and D (Skobe et al., 2001; Stacker et al., 2001). Activation of the VEGFR-3 signaling pathway is recognized to play an important role in tumor growth and metastasis by promoting lymphangiogenesis, and disruption of this pathway is a current strategy for the development of anticancer drugs (He et al., 2002; Karpanen et al., 2001;2006; Lin et al., 2005; Makinen et al., 2001; Ruggeri et al., 2003). Here we identified that CPX inhibition of LEC tube formation is attributed to suppressing VEGFR-3 protein expression. This is supported by the observations that i) CPX inhibited VEGFR-3 protein expression; ii) overexpression of VEGFR-3 conferred high resistance to CPX inhibition of LEC tube formation; and iii) downregulation of VEGFR-3 mimicked the effect of CPX, blocking LEC tube formation. These data are in agreement with previous findings that blocking VEGFR-3 signaling alone by VEGFR-3 fusion protein (Karpanen et al., 2001; 2006), VEGFR-3 soluble form (Lin et al., 2005; Makinen et al., 2001), or small-molecule inhibitors of VEGFR-3 (He et al., 2003; 2005; Ruggeri et al., 2003) inhibited lymphangiogenesis. It should be mentioned that we failed to detect VEGF-C/D in LECs even without CPX treatment (data not shown). This is consistent with the notion that VEGFs are generally produced and secreted by tumor cells, but not by the vascular or lymphatic endothelial cells (Alitalo and Carmeliet, 2002; Kerbel and Kamen, 2004). The results further support that CPX inhibition of LEC tube formation in our model was not through affecting VEGF-C/D expression, but is primarily through blocking VEGFR-3 protein expression. However, we also noticed that overexpression of VEGFR-3 failed to rescue the tube formation inhibited by CPX completely (Fig.2D), suggesting that CPX inhibits LEC tube formation involving more signaling molecules. More studies are needed to address this issue.

CPX has been reported to inhibit angiogenesis by blocking proline hydroxylation and maturation of collagen in HUVEC cells (Clement et al., 2002), but it has also been described to stimulate angiogenesis by inducing expression of hypoxia-inducible factor 1 α and VEGF in human liver carcinoma (HepG2) cells (Linden et al., 2003). Our preliminary studies indicate that treatment with CPX (5-10 μM, 24 h) downregulated protein expression of VEGF-C in prostate (PC-3) and breast (MDA-MB231) carcinoma cells (data not shown), which is in contrast to the findings in HepG2 cells. Probably this is due to the difference in cell lines or experimental conditions used. Further studies are needed to determine whether

CPX inhibits lymphangiogenesis *in vivo* by inhibiting both VEGFR-3 protein expression in LECs and VEGF-C/D expression in tumor cells.

Studies have shown that CPX affects expression of iron permeases or transporters (FTR1, FTR2, FTH1), a copper permease (CCC2), an iron reductase (CFL1), and a siderophore transporter (SIT1) in the fungus *Candida albicans* (Niewerth et al., 2003; Sigle et al., 2005), HIF-1α and VEGF in mammalian cells at transcriptional level (Linden et al., 2003). In this study, we found that CPX inhibited protein expression of VEGFR-3 in LECs. Therefore, we hypothesized that CPX inhibits VEGFR-3 protein expression by downregulating its mRNA level in LECs. However, unexpectedly, CPX did not alter VEGFR-3 mRNA level. Instead, CPX inhibited protein synthesis and promoted protein degradation of VEGFR-3 in the LECs. The results suggest that CPX may affect expression of different proteins through distinct mechanisms, and CPX controls VEGFR-3 protein expression at translational and post-translational levels.

It has been described that VEGFR-3 activates PI3K/Akt, ERK1/2 and JNK pathways (Wissmann and Detmar, 2006), promoting cell proliferation, migration and survival (Dixelius et al., 2003; Fournier et al., 1999; Salameh et al., 2005; Su et al., 2006). Here we found that CPX did not affect either cellular protein or phosphorylation levels of Akt, JNK and p38 MAPK, but potently inhibited phosphorylation of ERK1/2 in murine LECs, suggesting that the effects of CPX on these signaling pathways are cell-dependent. Furthermore, we identified that CPX inhibition of ERK1/2 phosphorylation was a consequence of downregulation of VEGFR-3 protein expression, since overexpression of VEGFR-3 attenuated CPX inhibition of phospho-ERK1/2, whereas downregulation of VEGFR-3 mimicked the effect of CPX, reducing phospho-ERK1/2 in the LECs. In addition, we also observed that expression of constitutively active MKK1 increased phospho-ERK1/2 and rendered high resistance to CPX inhibition of LEC tube formation. Selective suppression of ERK1/2 by U0126 (5 μM) directly blocked LEC tube formation by ~90%. In combination, CPX (5 μM) did not additively or synergistically enhance the inhibitory effect of U0126 (5 μM) on the tube formation, implying that ERK pathway is a critical controller for the LEC tube formation, and U0126 (5 μM) and CPX (5 μM) may redundantly inhibit ERK activation. We have not tested whether at lower concentrations, U0126 and CPX have any synergistic or additive inhibitory effect on tube formation. However, we noticed that expression of constitutively active MKK1 only conferred partial resistance to VEGFR-3 shRNA inhibition of LEC tube formation. In line with the observation shown in Fig.3B, downregulation of VEGFR-3 by 90% resulted in 10% LEC tube formation, whereas expression of MKK1-R4F in the VEGFR-3 shRNA-treated cells, about 40% LEC tube formation could be detected (data not shown). The results suggest that in addition to ERK1/2, there may be other signaling molecules involved in the VEGFR-3-mediated the tube formation in LECs as well. Our data are consistent with the recent findings that activation of ERK pathway promotes lymphoangiogenesis (Fritz-Six et al., 2008; Jin et al., 2008; Ming et al., 2009). Taken together, the results indicate that CPX inhibition of LEC tube formation at least partially through inhibition of VEGFR-3-mediated ERK1/2 pathway.

In summary, we have shown that CPX inhibited LEC tube formation in a concentration and time-dependent manner. CPX inhibition of the LEC tube formation was related to

suppression of VEGFR-3 protein expression, which occurred at translational and post-translational levels. Furthermore, our data suggest that CPX inhibition of LEC tube formation was in part by targeting VEGFR-3-mediated ERK pathway, and CPX may act as a novel anti-lymphangiogenic agent. The findings may facilitate repurposing the off-patent drug CPX for cancer therapy.

Materials and methods

Chemicals

CPX (Sigma, St. Louis, MO, USA) was dissolved in 100% ethanol to prepare a stock solution (100 mM), and then aliquoted and stored at −20°C. U0126, a selective inhibitor of MKK1/2, was obtained from LC Laboratories (Woburn, MA, USA).

Cell lines and cultures

Murine lymphatic endothelial cells (LECs) (Ando et al., 2005) were grown in antibiotic-free Dulbecco's modified Eagle medium (DMEM)/F12 medium (Mediatech, Herndon, VA, USA) supplemented with 10% FBS (Hyclone, Logan, UT, USA) at 37 °C and 5% CO_2. Human embryonic kidney (HEK) 293 (American Type Culture Collection, Manassas, VA), 293TD and 293A cells (Invitrogen, Carlsbad, CA, USA) were grown in antibiotic-free Dulbecco's modified Eagle medium (DMEM) (Mediatech) supplemented with 10% heat-inactivated FBS and non-essential amino acid (Mediatech) at 37°C and 5% CO_2.

Lentiviral shRNA cloning, production and infection

To generate lentiviral shRNA to VEGFR-3, oligonucleotides containing the target sequences were synthesized, annealed and inserted into FSIPPW lentiviral vector (Kanellopoulou et al., 2005) (a gift from Dr. Jun Chung, Louisiana State University Health Sciences Center, Shreveport, LA) through the EcoR1/BamH1 restriction enzyme site. The oligonucleiotides used were: VEGFR-3 sense: 5′-AATTCCCGCACTGCCACAAGAAGTACTGCAAGAGAGTACTTCTTGTGGCAG TGCTTTTTG-3′, antisense: 5′-GATCCAAAAAGCACTGCCACAAGAAGTACTCTCTT GCAGTACTTCTTGTGGCAGTGCGGG-3′. Lentiviral shRNA constructs targeting green fluorescence protein (GFP, as control) and VEGFR-3 were made as described (Liu et al., 2006). Subsequently, LEC cells, when grown to about 70% confluence, were infected with the above lentiviral shRNAs in the presence of 8 mg/ml polybrene, and exposed to 2 mg/ml puromycin after 24 h of infection. In 5 days, cells were used for experiments.

Plasmids and Transfection

Human VEGF-R3 deleted in its signal peptide was amplified via PCR from pCMV6-XL4-Flt4-TV1 (NM_182925; OriGene, Rockville, MD) encoding the full length VEGF-R3L, using the following oligonucleotides: AAAAAGCTTGGATCCAGTGACTACTCCATGACCCCC and AGCGGCCGCTTAGTAGCTGTTGTCTGTGAAG. The PCR product was first cloned into pTopo (Invitrogene) and sequenced. Subsequently, the product was digested with the restriction enzymes HindIII and EcoRV, and cloned into the same sites of p3×Flag-CMV9 (Sigma) in the frame with the signal peptide and the 3×Flag Tag to generate p3×Flag-

VEGFR-3-TV1. LECs (5×10^5 cells) in 6-well plates were transfected with the plasmid p3×Flag-VEGFR-3-TV1 using Lipfectamine™ 2000 reagent (Invitrogen) per manufacture's instructions. Empty vector was used as a control. After transfection for 48 h, cells were transferred to 100-mm dishes and cultured for 2 weeks at 37°C 5% CO_2 in the growth medium containing 500 μg/ml geneticin (G418 sulfate; Invitrogen). Drug-resistant clones were screened by Western blot analysis for overexpression of VEGFR-3. Finally, desired LEC clones stably overexpressing VEGFR-3 were pooled and expanded in growth medium containing G418 (500 μg/ml).

Recombinant adenoviral constructs and infection of cells

The recombinant adenoviruses expressing GFP (Ad-GFP) and Flag-tagged constitutively active MKK1 (Ad-MKK1-R4F) were described previously (Liu et al., 2008; 2010). All adenoviruses were amplified, titrated and used as described (Liu et al., 2006).

Western blot analysis

Western blot analysis was performed as described previously (Liu et al., 2006). The primary antibodies used included antibodies to VEGFR-3, GAPDH, Akt, ERK2, JNK1, phospho-JNK (Thr183/Tyr185), p38, phospho-p38 (Thr180/Tyr182), MKK1, Flag (Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho-ERK1/2 (Thr202/Tyr204), phospho-Akt (Ser473) (Cell Signaling, Beverly, MA, USA) and β-tubulin (Sigma).

Cell morphological analysis

LECs were seeded at a density of 5×10^5 cells/well in a 6-well plate. Next day, the cells were treated with CPX (0-5 μM) for 24 h, or with 5 μM CPX for 0-24 h, followed by taking images with an Olympus inverted phase-contrast microscope (Olympus Optical C., Melviller, NY, USA) (200×) equipped with the Quick Imaging system.

Tube formation assay

Tube formation assay was performed as described (Schacht et al., 2003). Briefly, LECs were harvested after indicated treatments, and then seeded into a 96-well plate (2×10^4/well) pre-coated with 40 μl (10 mg/ml) growth factor-reduced matrigel (BD Biosciences, Billerica, MA, USA). After 2-3 h incubation at 37°C, the capillary tube structures were observed and representative images were captured with an Olympus inverted phase-contrast microscope (Olympus Optical C., Melviller, NY, USA) (200×) equipped with the Quick Imaging system. Tube length was quantified by NIH Image J.

Semi-quantitative RT-PCR

Total mRNA was extracted from LECs using TRIzol® Reagent (Invitrogen) following the manufacture's instruction. cDNA was constructed using MML-VII reverse transcriptase (Invitrogen) and oligo $(dT)_{12\text{-}18}$ primer (Invitrogen). PCR was performed using 10 ng cDNA, Tag DNA polymerase (New England Biolabs, Ipswich, MA, USA) and specific primer pairs for VEGFR-3 and β-actin (as internal control), respectively. The forward primer set for VEGFR-3 is: 5′-CAGGATGAAGACA TTTGAGGA-3; and the backward primer is: 5′-TGAAGCCGCTTTC TTGTCTATG-3; the forward primer set for β-actin is: 5′-

TGACGGGGTCACCCACACTGTGCCCAT; and the backward primer is: 5′-CTAGAAGCATTTGCGGT GGACGATGGA GGG. The amplification was done for 30 cycles (94°C 30s, 60°C 30s, and 72°C 40s). PCR products were size-fractionated on 2% agarose gels and visualized on a UV transilluminator.

Protein synthesis and degradation assay

To determine the effect of CPX on protein synthesis of VEGFR-3, *in vivo* ^{35}S-Met/Cys labeling (Sunavala-Dossabhoy et al., 2004) was used. Briefly, LECs grown in 100-mm dishes in the growth medium were pretreated with CPX (0-5 μM) for 24 h or for 0-24 h at 5 μM. Subsequently, the cells were washed with PBS twice, switched to 3 ml Met/Cys-free DMEM (Mediatech) containing 10 μM MG-132 (Calbiochem, Gibbstown, NJ, USA), a proteasome inhibitor, and then pulsed with 0.3 mCi/ml ^{35}S-Met/Cys (MP Biomedicals, Solon, OH, USA) for up to 4 h, followed by immunoprecipitation with antibodes to VEGFR-3 and GAPDH (as control), respectively, in the presence of proteinA/G agarose (Santa Cruz Biotechnology) overnight at 4°C. The immunocomplexes were harvested, separated by SDS-PAGE, transferred to 0.22 μm PVDF membrane (Santa Cruz Biotechnology), and then exposed to x-ray film for autoradiography.

To determine the effect of CPX on protein degradation, LECs were grown in the growth medium and treated with 50 μg/ml cycloheximide (CHX, Sigma) with or without CPX (5 μM) for 0-12 h. Subsequently, cell lysates were collected, followed by Western blot analysis with antibodies to VEGFR-3 and β-tubulin (as control), respectively.

Statistical analysis

Results were expressed as mean values ± standard deviation (mean ± SD). The data were analyzed by one-way analysis of variance (ANOVA) followed by post-hoc Dunnett's *t*-test for multiple comparisons. A level of $P < 0.05$ was considered to be significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by NIH grant CA115414 (S. H.), Louisiana Board of Regents grant [LEQSF(2006-09)-RD-A-18] (S. H.), and American Cancer Society grant RSG-08-135-01-CNE (S.H.). We thank Dr. Natalie Ahn for providing MKK1 constructs.

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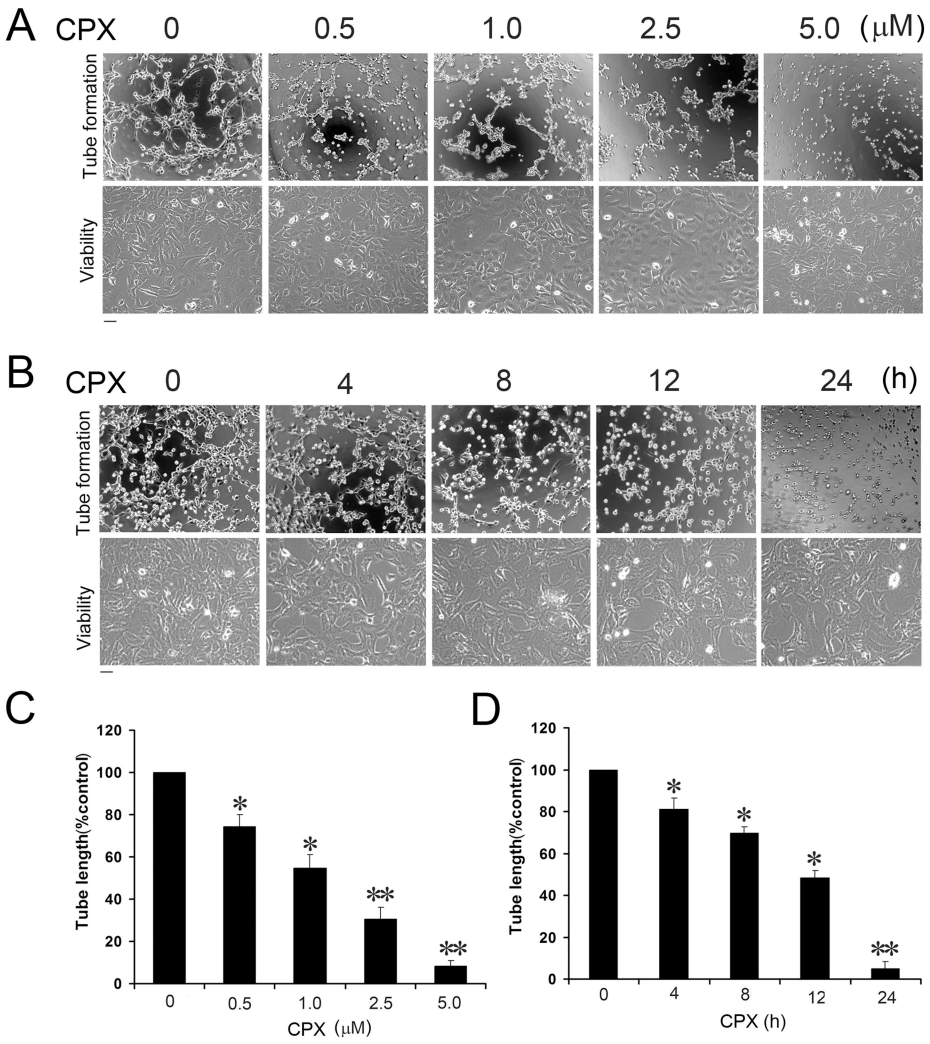

Figure 1. CPX inhibits LEC tube formation in a concentration and time-dependent manner

LECs were treated with CPX (0-5 μM) for 24 h, or CPX (5 μM) for 0-24 h, followed by tube formation assay and morphological analysis, as described in "Materials and methods". Representative images are shown in (A) and (B), respectively. Bar = 100 μm. The length of tube-like formation was evaluated by NIH Image J software. Quantitative data are presented as mean ± SD (n = 3) in (C) and (D), respectively. **P* < 0.05, ***P* < 0.01, difference *vs*. control group.

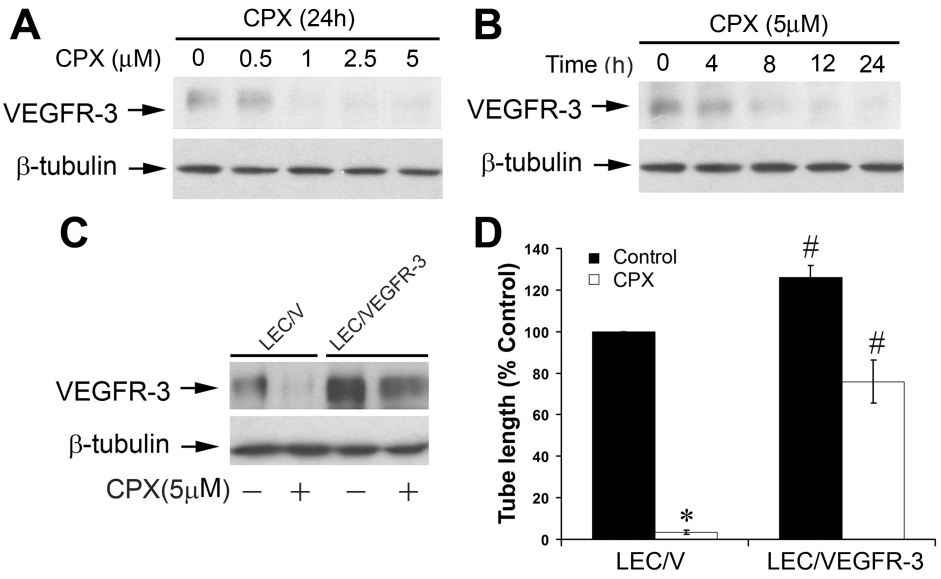

Figure 2. CPX inhibition of LEC tube formation is associated with suppressing VEGFR-3 protein expression

A and B, CPX inhibited protein expression of VEGR-3 in a concentration and-time-dependent manner. LECs, treated with CPX (0-5 μM) for 24 h (A) or CPX (5 μM) for 0-24 h (B), were harvested and subjected to Western blot analysis with antibodies to VEGFR-3. β-tubulin was used as a loading control. C and D, Overexpression of VEGFR-3 partially prevented CPX inhibition LEC tube formation. LEC/V (control) and LEC/VEGFR-3 cells were treated with CPX (5 μM) for 24 h, followed by Western blot analysis with indicated antibodies (C), or tube formation assay as described in "Materials and methods" (D). Quantitative results of tube formation are shown as mean ± SD (n = 3) in (D). **P* < 0.05, difference *vs*. control group; #*P* < 0.05, difference *vs*. LEC/V group.

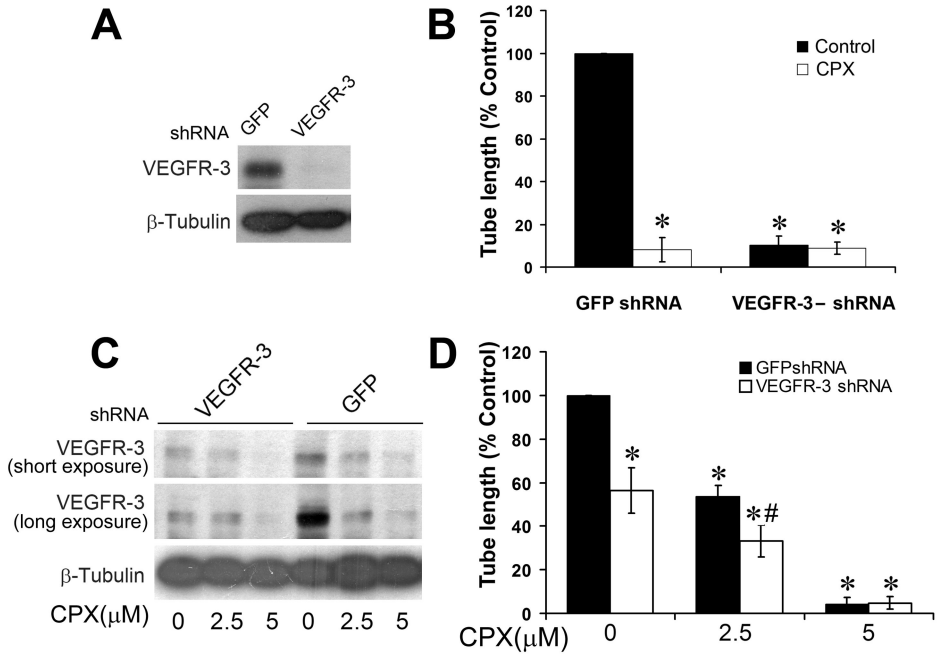

Figure 3. Downregulation of VEGFR-3 mimicks the effect of CPX, blocking LEC tube formation

Lentiviral shRNA to VEGFR-3, but not GFP, downregulated VEGFR-3 protein expression by ~90% in LECs, as detected by Western blotting (A). LECs, infected with lentiviral shRNAs to VEGFR-3 and GFP (control), respectively, were treated with CPX (5 μM) for 24 h, followed by tube formation assay as described in "Materials and methods". Quantitative results of tube formation are shown as mean ± SD (n = 3) in (B). **P* < 0.05, difference *vs*. GFP shRNA control group. C and D, LECs, infected with half amount of lentiviral shRNA to VEGFR-3 and GFP (control), respectively, were treated with/without CPX (0, 2.5 and 5 μM) for 24 h, followed by Western blotting (C) and tube formation assay (D). Quantitative results of tube formation are shown as mean ± SD (n = 3). **P* < 0.05, difference *vs*. GFP shRNA.

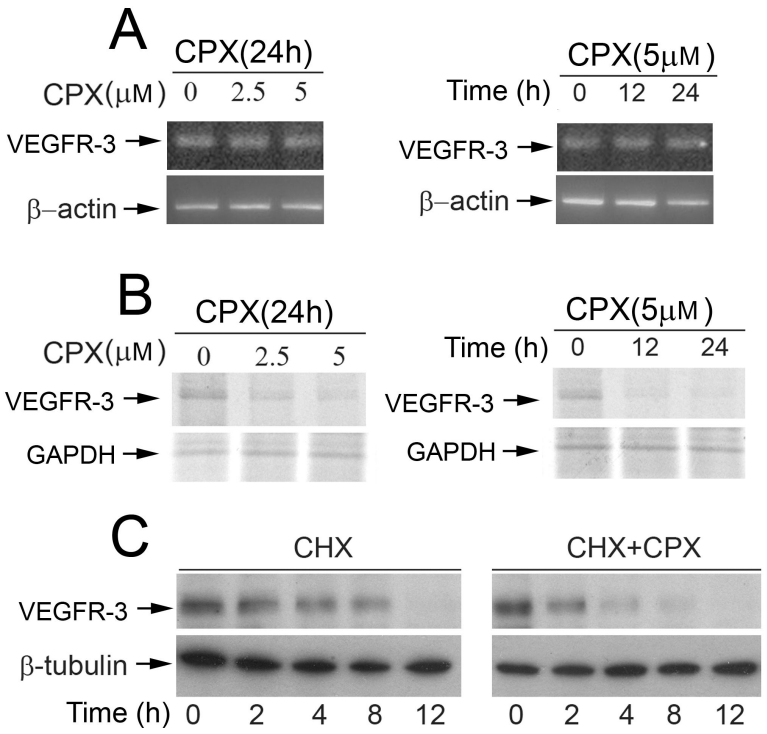

Figure 4. CPX does not alter mRNA expression, but inhibits protein synthesis and promotes protein degradation of VEGFR-3

A, CPX did not affect VEGFR-3 mRNA level. Total RNA was extracted from LECs treated with CPX (0-5 μM) for 24 h (Left panel) or with CPX (5 μM) for 0-24 h (Right panel), followed by semi-quantitative RT-PCR. β-actin was used as a loading control. B, CPX inhibited protein synthesis of VEGFR-3 in LECs. LECs were pretreated with CPX (0-5 μM) for 24 h (Left panel) or with CPX (5 μM) for 0-24 h (Right panel), and then pulsed with ^{35}S-Met/Cys for 4 h, followed by immunoprecipitation with antibodies to VEGFR-3. The immunoprecipitates were separated by SDS-PAGE and transferred to PVDF membranes, followed by autoradiography. GAPDH served as an internal control. C, CPX promoted protein degradation of VEGFR-3 in LECs. LECs, grown in 10% FBS-DMEM medium, were exposed to cycloheximide (CHX, 50 μg/ml), in the presence or absence of CPX (5 μM), for 0-12 h, followed by Western blot analysis with indicated antibodies.

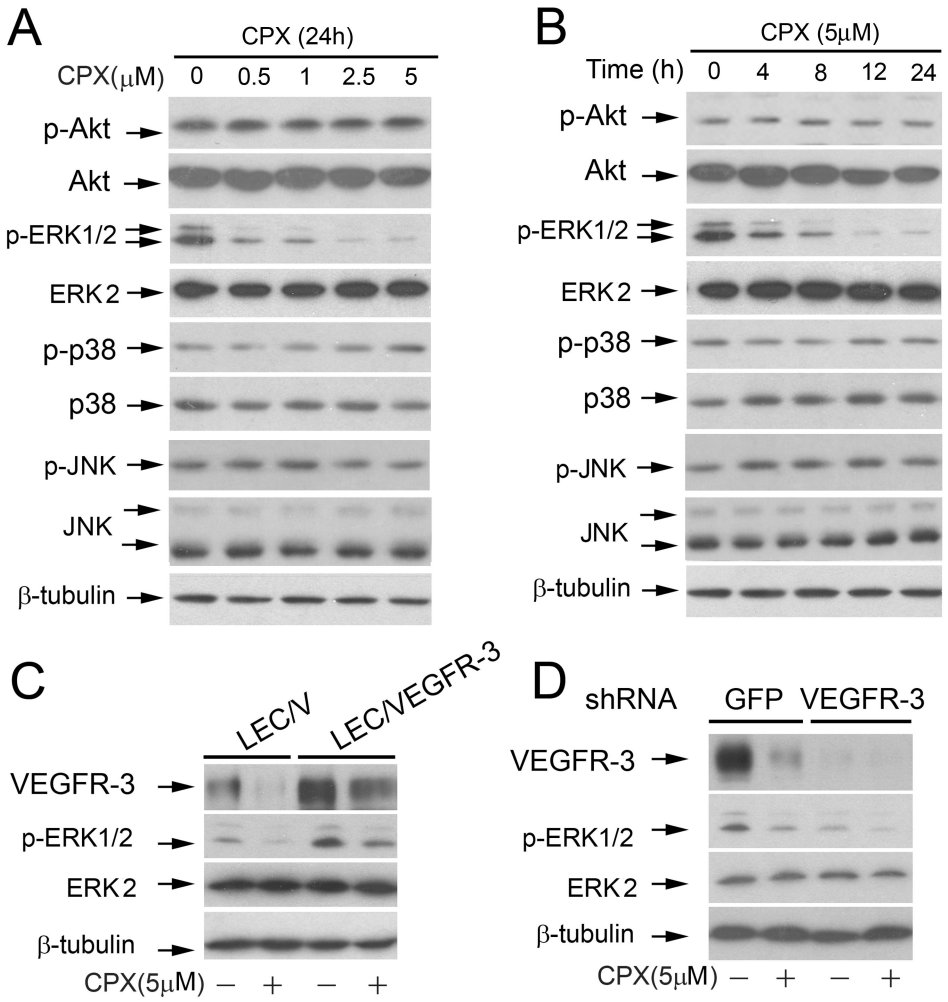

Figure 5. CPX inhibits VEGFR-3-mediated ERK1/2 pathway

A and B, CPX inhibited phosphorylation of ERK1/2, but not Akt, JNK and p38 MAPK, in LECs in a concentration and time-dependent manner. LECs, treated with CPX (0-5 μM) for 24 h (A) or CPX (5 μM) for 0-24 h (B), were harvested and subjected to Western blot analysis with indicated antibodies. β-tubulin was used as a loading control. C, Overexpression of VEGFR-3 conferred resistance to CPX inhibition of ERK1/2 phosphorylation in LECs. LEC/V (control) and LEC/VEGFR-3 were treated with or without CPX (5 μM) for 24 h, followed by Western blotting with indicated antibodies. D. Downregulation of VEGFR-3 mimicked the effect of CPX, inhibiting phosphorylation of ERK1/2 in LECs. LECs, infected with lentiviral shRNAs to VEGFR-3 and GFP (control), respectively, were treated with CPX (5 μM) for 24 h, followed by Western blotting with indicated antibodies.

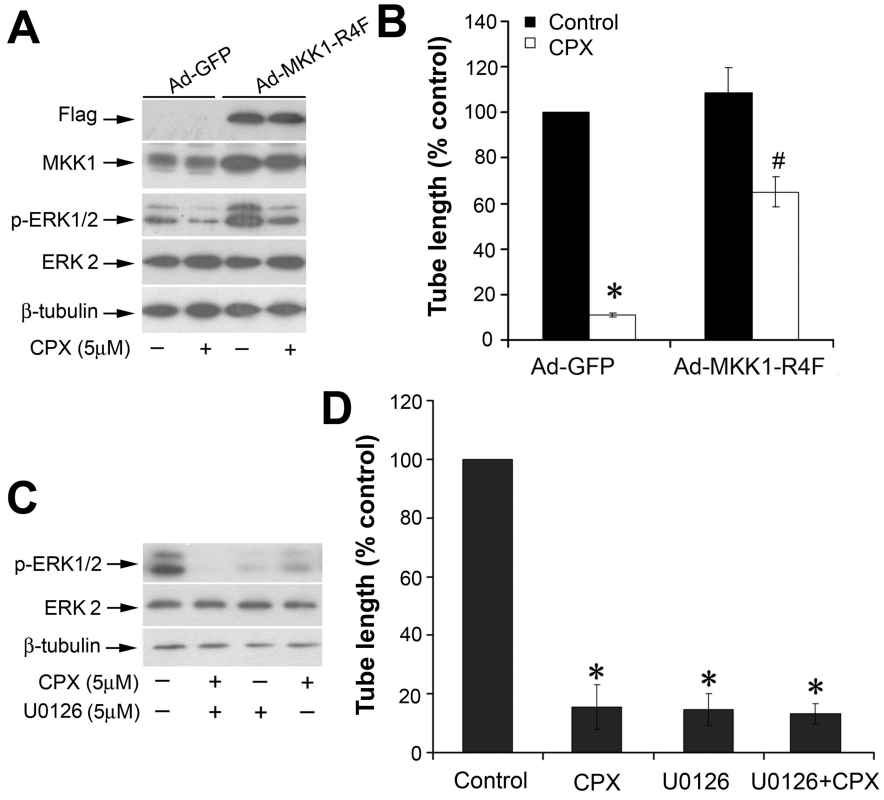

Figure 6. CPX inhibition of LEC tube formation is through targeting VEGFR-3-mediated ERK1/2 pathway

A and B, Expression of constitutively active MKK1 attenuated CPX inhibition of ERK1/2 phosphorylation and the tube formation in LECs. LECs, infected with Ad-MKK1-R4F and Ad-GFP (control), respectively, were treated with or without CPX (5 μM) for 24 h, followed by Western blotting with indicated antibodies (A), or by tube formation assay as described in "Materials and methods" (B). Quantitative results are shown as mean ± SD (n = 3) in (B). *$P < 0.05$, difference *vs*. control group; $^{\#}P < 0.05$, difference *vs*. Ad-GFP group. C and D, In combination with U0126, CPX did not enhance U0126 inhibition of LEC tube formation. LECs were treated with U0126 (5 μM) or CPX (5 μM) alone, or both for 24h, followed by Western blotting using the indicated antibodies (C), or by tube formation assay as described in "Materials and methods" (D). Quantitative results of tube formation are shown as mean ±SD (n = 3). *$P < 0.05$, difference *vs*. control group.