Shear stress and VEGF activate IKK via the Flk-1/Cbl/Akt signaling pathway

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Wang, Yingxiao, Joann Chang, Yi-Chen Li, Yi-Shuan Li, John Y.-J. Shyy, and Shu Chien. Shear stress and VEGF activate IKK via the Flk-1/Cbl/Akt signaling pathway. Am J Physiol Heart Circ Physiol 286: H685–H692, 2004. First published October 9, 2003; 10.1152/ajpheart.00237.2003.—Vascular endothelial cells are continuously exposed to mechanical (e.g., shear stress) and chemical (e.g., growth factors) stimuli. It is important to elucidate the mechanisms by which cells perceive and integrate these different stimuli to regulate the downstream signaling pathways. We (50) have previously reported the shear-induced interplay between two membrane receptors, integrins and Flk-1. In the present study, we investigated the molecular mechanisms regulating the downstream IκB kinase (IKK) pathway in response to shear stress and VEGF. Both shear stress and VEGF induced a transient increase of IKK activity. These effects were inhibited by SU-1498, a specific Flk-1 inhibitor, and by a negative mutant of Casitas B-lineage lymphoma (Cbl) with tyrosine-to-phenylalanine mutations at sites 700, 731, and 774 (Cblmut). Because Flk-1 and Cbl form a complex upon shearing or VEGF applications (50), these results suggest that shear stress and VEGF activate IKK via the receptor Flk-1 and its recruitment of the adapter protein Cbl. The inhibition of the shear- and VEGF-induced IKK activities by a negative mutant of Akt indicates that Akt acts upstream to IKK in response to shear stress and VEGF. Furthermore, SU-1498 and Cblmut abolished the shear- and VEGF-induced Akt activity, indicating that Akt acts at a level downstream to Flk-1 and Cbl. Therefore, our results indicate that the signaling events induced by shear stress and VEGF converge at the membrane receptor Flk-1 and that these stimuli share the Flk-1/Cbl/Akt pathway in activating IKK activation.

As a membrane receptor, Flk-1 belongs to the receptor tyrosine kinase (RTK) family. It has been well documented that Flk-1 serves as a major receptor to modulate functional signaling pathways in response to VEGF, by functioning as the kinase for many substrates and by recruiting signaling molecules (38). As an adapter protein associated with Flk-1, Casitas B-lineage lymphoma (Cbl) plays a central role in tyrosine kinase-related signal transduction (35, 44). There is ample evidence that Cbl associates with not only RTKs (e.g., EGF receptor, PDGF receptor, and colony-stimulating factor-1 receptor) but also other adapter proteins, e.g., p85, Grb2, Shc, Crk, and Nck (35). This unique property provides Cbl with the potential of being a key molecule in regulating multiple signaling pathways.

The Cbl-associated adapter protein p85 is a subunit of the lipid kinase phosphatidylinositol 3-kinase (PI3K), which has been well documented to regulate the serine/threonine protein kinase Akt (6, 13, 25). The activation of Akt involves the binding of the phosphoinositide products of PI3K to the pleckstrin homology domain of Akt and the consequent Akt translocation to the plasma membrane, where it is presented to upstream activating kinases. The subsequent phosphorylations of Thr308 and Ser473 on Akt lead to its activation. Whereas Thr308 is phosphorylated by phosphoinositide-dependent kinase 1 and is essential for Akt activation, the phosphorylation at Ser473 appears to be dependent on PI3K and is an indication of the maximal activation of Akt (1, 4, 8, 43). Although the precise molecular mechanism of Ser473 phosphorylation remains to be elucidated, it is generally accepted that it involves Akt autophosphorylation as well as phosphorylation by phosphoinositide-dependent kinase 2 (8). Recent studies (32, 39, 42) have indicated that Akt may form a complex with IκB kinase (IKK) and subsequently protect cells from apoptosis. IKK regulates the transcriptional factor NF-κB, which modulates the expression of many genes involved in inflammatory responses, cell growth, and death (2, 23). Upon stimulation, the activated IKK phosphorylates IκB proteins, leading to the ubiquination and degradation of IκB. Because NF-κB is sequestered in the cytoplasm through its tight association with inhibitory IκB proteins, the degradation of IκB results in the release of NF-κB and its translocation into the nucleus to turn on the expression of multiple genes (2, 22). Although both shear stress and VEGF have been reported to induce the activations of IκB and NF-κB (5, 20, 24, 34), it is unclear as to the underlying signaling pathways leading to IKK activation in response to shear stress or VEGF.

In the present study, we investigated the molecular mechanisms by which shear stress and VEGF activate the IKK pathway. Our results showed that both shear- and VEGF-

VASCULAR ENDOTHELIAL CELLS (ECs) are continuously exposed to mechanical (e.g., shear stress) and chemical (e.g., VEGF) stimuli, which are important modulators of vascular cell functions in normal and pathophysiological conditions (7, 16, 45, 52). Shear stress has been shown to activate multiple membrane receptors, e.g., integrins, VEGF receptor (VEGFR)-2 (Flk-1), G protein-coupled receptors, and ion channels (3, 40), and modulate downstream signaling molecules, including focal adhesion kinase (FAK) (28), Src (19), Ras (29), and Rho GTPases (27). As a growth factor, VEGF activates its receptors Flk-1 (VEGFR-2) and Flt-1 (VEGFR-1). The activation of Flk-1 can lead to the binding and phosphorylation of adapter proteins, such as Shc and Nyc, and the activation of downstream signaling pathways (26, 41). Although there is evidence indicating that shear stress and VEGF can activate similar signaling molecules, it remains unclear as to whether these two types of stimuli share the same receptor-adapter machinery to regulate the downstream signaling pathways.

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induced activations of IKK require the mediators by Flk-1 and Cbl and subsequently Akt. Therefore, mechanical and chemical stimuli may converge at the receptor Flk-1 to utilize a common network of adapter and signaling molecules to regulate cellular functions.

MATERIALS AND METHODS

Cell culture. Cell culture reagents were obtained from GIBCO-BRL (Grand Island, NY). Bovine aortic ECs (BAECs) were isolated from the bovine aorta with collagenase and cultured in a humidified 95% air-5% CO₂ incubator at 37°C. The culture medium was DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 U/ml penicillin, 100 µg/ml streptomycin, and 1 mM sodium pyruvate. All experiments were conducted with BAEC cultures before passage 10.

Shear stress and VEGF treatment. A flow system was used to impose shear stress on the cultured ECs as described by Frangos et al. (12). In all experiments, BAECs were starved for 12 h in 0.5% serum and subsequently for 2 h in serum-free medium before they were subjected to shear stress (12 dyn/cm²) or VEGF (10 ng/ml).

DNA plasmids and transient transfection. The expression plasmid hemagglutinin (HA)-IKK-β, which encodes HA epitope-tagged IKK-β, has been described previously (55). The HA epitope-tagged wild-type Akt plasmid (HA-Aktwt) was constructed in the vector pCMV. For the dominant negative mutant of Akt (HA-Aktmut), the major phosphorylation sites (Thr³⁰⁸ and Ser⁷³) in HA-Aktwt were mutated into alanine. HA epitope-tagged wild-type Cbl (HA-Cblwt) and HA epitope-tagged negative mutant of Cbl (HA-Cblmut) were gifts from Dr. Alexander Y. Tsygankov. In HA-Cblmut, tyrosine to phenylalanine mutations were introduced at positions 700, 731, and 774, creating sites for NF-κB/H9260 from Dr. Alexander Y. Tsygankov. In HA-Aktmut, tyrosine to phenylalanine mutations were introduced at positions 700, 731, and 774, which are the major tyrosine phosphorylation sites of c-Cbl (11). HIV(LTR)-Luc encodes a luciferase reporter driven by the human immunodeficiency virus long terminal repeat that contains two binding sites for NF-κB (37). The pSV-β-galactosidase plasmid contains a β-galactosidase gene driven by the simian virus 40 promoter and enhancer. The various plasmids were transfected into BAECs at 80% confluency using the lipofectamine method as described by the manufacturer (GIBCO-BRL).

Immunoprecipitation and immunoblotting. The antibodies used for immunoprecipitation and immunoblotting were polyclonal anti-phospho-Akt(Thr³⁰⁸) (Cell Signaling), polyclonal anti-Akt, and polyclonal anti-HA antibodies (Santa Cruz Biotechnology; Santa Cruz, CA). For immunoprecipitation, the cells were lysed in a buffer containing 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 5 mM NaF, 1 mM Na₂VO₃, 1 mM PMSF, and 10 µg/ml leupeptin. The lysate was centrifuged, and the supernatant was immunoprecipitated with appropriate antibodies and protein A-Sepharose beads (Amersham Pharmacia Biotech) at 4°C overnight. The immunoprecipitated complexes were washed and used for either kinase activity assays or immunoblotting. For immunoblotting, proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane. The membrane was then blocked with 5% bovine serum albumin, followed by incubation with the primary antibody. The bound primary antibodies were detected by using a goat anti-rabbit IgG-horseradish peroxidase conjugate (Santa Cruz Biotechnology) and the ECL detection system (Amersham Pharmacia Biotech).

Kinase activity assays. Various plasmids as indicated were transfected into BAECs with HA-IKK-β or HA-Aktmut, which were immunoprecipitated from the cell lysate using protein A-Sepharose beads and an anti-HA polyclonal antibody. The immunocomplex was resuspended in the buffer containing [γ-³²P]ATP and an appropriate substrate, i.e., glutathione-S-transferase (GST)-IκB-α(1–54) for IKK kinase assay (55) and histone 2B (H2B) for Akt kinase assay (53). The kinase reaction mixture was resolved by SDS-PAGE after incubation at 30°C for 30 min, and phosphorylated GST-IκB-α or H2B was detected by autoradiography.

RESULTS

Shear stress and VEGF induced a transient activation of IKK. We (5) have previously shown that shear stress can induce the activation of IKK, which was assayed as a combination of IKK-α and IKK-β. Because IKK-β, but not IKK-α, has been reported to be critical in the activation of NF-κB (30), in the present study we determined the effect of shear stress on IKK-β. BAECs were transfected with HA-IKK-β and subjected to various time periods of shear stress (12 dyn/cm²) or kept as static control. The kinase activity assay revealed that shear stress induced IKK-β activity as early as 1 min, peaked at 5 min, and returned to the basal level at 15 min (Fig. 1A). The results indicate that shear stress induced transient IKK-β activity.

VEGF has been previously reported to cause the phosphorylation of IκB-α and the activation of NF-κB (20, 24, 34). To test the effect of VEGF on IKK-β activation, BAECs were transfected with HA-IKK-β and treated with the Flk-1 ligand VEGF (10 ng/ml) for various time periods. Similar to shear...
stress, VEGF also initiated a rapid and transient increase of IKK-β activity, with a peak at 5 min (Fig. 1B).

These results show that both mechanical (shear stress) and chemical (VEGF) stimuli can induce transient IKK-β activation in BAECs.

**Flk-1 and Cbl mediate the activation of IKK induced by shear stress and VEGF.** We (50) have shown that both shear stress and VEGF induce Flk-1-Cbl association. Because both Cbl and IKK play important roles in inflammatory responses (2, 35) and Flk-1 activation has been implicated in the activation of the IKK/NF-κB signaling pathway in a variety of cell types (20, 24, 34, 51), we tested the hypothesis that Flk-1 and Cbl mediate the effects of shear stress and VEGF on the IKK signaling pathway, possibly by forming a Flk-1-Cbl association, with Cbl serving as an adapter protein to provide multiple docking sites for downstream signaling molecules.

We first examined the role of Flk-1 in mediating shear-induced IKK activation. BAECs were transfected with HA-IKK-β and then treated with SU-1498 (a specific inhibitor of Flk-1) or DMSO (solvent control) before being subjected to shear stress for 5 min. HA-IKK-β was immunoprecipitated with anti-HA antibody and subjected to an in vitro kinase assay with GST-IκB-α as the substrate. As shown in Fig. 2A, SU-1498 significantly attenuated shear-induced IKK activity. We then asked the question of whether Cbl, as an adapter protein with multiple docking sites, was also involved in the regulation of shear-induced IKK activation. We did not observe significant differences in IKK activities between BAECs transfected with HA-Cblwt and PCDNA3, an empty vector, suggesting that endogenous Cbl is sufficient to mediate the effects of shear stress and VEGF (data not shown). Therefore, HA-Cblwt was chosen as the control for HA-Cblnm in assessing the effect of Cbl on IKK activation. HA-IKK-β was cotransfected with HA-Cblwt or HA-Cblnm into BAECs. As shown in Fig. 2B, HA-Cblnm markedly attenuated shear-induced IKK activity compared with control cells transfected with HA-Cblwt. This result suggests that Cbl plays a significant role in shear activation of IKK.

We further investigated the role of Flk-1 and Cbl in mediating VEGF activation of IKK. HA-IKK-β-transfected BAECs were treated with SU-1498 or DMSO before being subjected to VEGF (10 ng/ml) for 5 min. As shown in Fig. 3A, SU-1498 blocked VEGF-induced IKK activation. To test the effects of Cbl on IKK activation in response to VEGF, HA-IKK-β was cotransfected with HA-Cblwt or HA-Cblnm into BAECs before VEGF stimulation. HA-Cblnm inhibited VEGF-induced IKK activation (Fig. 3B). These results suggest that both Flk-1 and Cbl play significant roles in VEGF activation of IKK.

These results indicate that both shear stress and VEGF activate IKK via Flk-1 and Cbl.

**Akt mediates the activation of IKK induced by shear stress and VEGF.** Akt has recently been reported to form a physical complex with IKK and regulate cell proliferation via the IKK/NF-κB pathway (39, 42). To examine the role of Akt in mediating shear and VEGF-induced activation of IKK, HA-IKK-β was cotransfected with HA-Aktwt or HA-Aktnm into BAECs before they were subjected to shear stress or VEGF. Kinase assays revealed that HA-Aktnm abolished shear- and VEGF-induced IKK activity (Fig. 4, A and B). Therefore, Akt activation is essential for both shear- and VEGF-induced activation of IKK.

**Flk-1 and Cbl mediate the activation of Akt induced by shear stress and VEGF.** After showing that Flk-1, Cbl, and Akt are all upstream molecules in shear and VEGF activation of IKK, we further examined their sequential order of action. Because Akt is a well-known effector of PI3K (10), which serves as a major Cbl-associated protein (35, 44), we hypothesized that Akt mediates the effect of Flk-1 and Cbl on shear-induced IKK activation by providing a linkage between Cbl and IKK.

To assess the involvement of Flk-1 in the shear-induced Akt activation, BAECs were incubated with SU-1498 or DMSO as
control for 1 h before being subjected to shear stress. As shown in Fig. 5A, SU-1498 abolished the shear-induced activation of Akt, as assessed by the phosphorylation of Ser473 in Akt. To examine the role of Cbl in regulating Akt, HA-Aktwt was cotransfected with HA-Cblwt or HA-Cblnm into BAECs. The exogenous Akt protein was pulled down by immunoprecipitation with anti-HA antibodies to perform the Akt kinase assay with H2B as the Akt substrate. Kinase assay revealed that HA-Cblnm markedly inhibited shear-induced Akt activation compared with cells transfected with HA-Cblwt (Fig. 5B), suggesting that Cbl is upstream of Akt in response to shear stress. Therefore, the results indicate that Akt acts at a level downstream of Flk-1 and Cbl while upstream of IKK in response to shear stress.

We further examined whether Flk-1, Cbl, and Akt also form a similar signaling hierarchy in response to VEGF. As shown in Fig. 6A, inhibition of Flk-1 by SU-1498 abolished VEGF-induced activation of Akt. Kinase assay also revealed that inhibition of Cbl with HA-Cblnm blocked Akt activation (Fig. 6B). These results suggest that Flk-1, Cbl, and Akt mediate VEGF-induced IKK activation in the same sequential order as in the case of shear-induced IKK activation (Fig. 7).
DISCUSSION

Different types of stimuli can regulate signaling pathways via convergent or divergent signaling pathways. For instance, both shear stress and PDGF activate ERK via a convergent

![Image](H689_CBL_AKT_SIGNALING_IN_IKK_ACTIVATION_BY_SHEAR_AND_VEGF)

**Fig. 5.** Flk-1 and Cbl mediate the activation of Akt induced by shear stress. A: BAECs were treated with DMSO (0.1%) or SU-1498 (5 μM) for 1 h before being subjected to shear stress for 5 min or static incubation. The cell lysates were immunoblotted with an anti-phospho-Akt(473) antibody to detect the phosphorylation of Ser^473 in Akt. B: HA-Akt_{wt} was cotransfected with HA-Cbl_{wt} or HA-Cbl_{nm} into BAECs. The transfected cells were subjected to shear stress for 5 min or static incubation. The cell lysates were immunoprecipitated with an anti-HA antibody for immunocomplex kinase assays using histone 2B (H2B) as the substrate. The top gel bands are phosphorylated Akt (A) or H2B (B), which indicate the level of Akt activation, and the bottom gel bands show IB with an anti-Akt (A) or anti-HA antibody (B), which indicate that the levels of the loaded proteins (A) or the expressed exogenous HA-tagged proteins (B) were comparable among the various samples. Bar graphs are results of densitometric analysis showing means ± SD of three separate experiments. #Significant differences (P < 0.05) between the SU-1498-treated sample and DMSO control after shearing in A and between cells transfected with HA-Cbl_{wt} and HA-Cbl_{nm} after shearing in B.

**Fig. 6.** Flk-1 and Cbl mediate the activation of Akt induced by VEGF. A: BAECs were treated with DMSO (0.1%) or SU-1498 (5 μM) for 1 h before being subjected to VEGF for 5 min or kept as control. The cell lysates were immunoblotted with an anti-phospho-Akt(473) antibody to detect the phosphorylation of Ser^473 in Akt. B: HA-Akt_{wt} was cotransfected with HA-Cbl_{wt} or HA-Cbl_{nm} into BAECs. Transfected cells were subjected to VEGF for 5 min or kept as control. The procedures for IP and IB, and the bands indicated by the arrows, are the same as those described in Fig. 5. Bar graphs are results of densitometric analysis showing means ± SD of three separate experiments. #Significant differences (P < 0.05) between the SU-1498-treated sample and DMSO control after VEGF challenge in A and between cells transfected with HA-Cbl_{wt} and HA-Cbl_{nm} after VEGF challenge in B.

PI3K signaling pathway (17, 33), whereas shear stress differentially regulates JNK and ERK via G\textsubscript{βγ} and G\textsubscript{i2}, respectively (21). Our present findings indicate that shear stress and VEGF share the Flk-1/Cbl/Akt signaling pathway in regulating IKK. Therefore, mechanical and chemical stimuli can converge at specific receptors and share common signaling pathways to
regulate cellular functions. Because living cells continuously receive both mechanical and chemical stimuli, this paradigm allows the cell to regulate its functions with limited signaling pathways in response to different external cues.

Many signal transducing molecules concentrate in the focal adhesion complex that connects the cytoskeleton to integrins and the extracellular matrix; hence, integrins and the focal adhesion complex may integrate mechanical signals and convert them into biochemical events (18). Indeed, our recent study (50) provided evidence that the activation of integrins leads to Flk-1/Cbl association and that the shear-induced ligand-independent Flk-1/Cbl association is dependent on integrins and cytoskeletal integrity. VEGF, as a ligand, binds to its transmembrane receptor Flk-1 on the cell surface. This ligand-receptor coupling results in an integrin-independent autophosphorylation of Flk-1 and the subsequent recruitment of Cbl (50). Hence, the signaling events induced by mechanical (shear stress) and chemical (VEGF) stimuli may converge at the same receptor Flk-1 despite their different mechanisms of receptor activation.

The present results indicate that Cblnm blocked IKK and Akt activations in response to shear stress and VEGF, whereas Cblwt had no detectable effect (Figs. 2B, 3B, 5B, and 6B). This is consistent with previous reports suggesting that Cbl serves as a positive regulator for Met-induced MAPK activation as well as IL-4-induced survival signals and PI3K activation (14, 48). Overexpression of Cbl, however, results in ligand-induced RTK degradation and internalization (31, 36, 49, 54). It is possible that Cbl may cause RTK internalization and degradation after the activation of downstream signals. This negative feedback mechanism may also explain the transient kinetics of shear- and VEGF-induced IKK activations (Fig. 1). Interestingly, the Cbl negative mutant had no detectable effect on shear-induced Flk-1-Cbl association (data not shown). Given that the primary binding site for p85 (Tyr731 in Cbl) has been removed in Cblnm, we postulate that overexpression of Cblnm can abolish the association between p85 and Cbl and hence decouple PI3K from the induced Flk-1-Cbl complex without perturbing the binding between Flk-1 and Cbl, which involves the tyrosine phosphorylation of Flk-1 and an intact phosphotyrosine binding domain in Cblnm (35, 44). This action in turn blocks the activation of PI3K and its downstream effector Akt, thus ultimately leading to the inhibition of IKK. This result indicates that Cblnm affects IKK activation at a level downstream to Flk-1/Cbl association to act on IKK/NF-κB (32, 39, 42).

In view of the facts that Flk-1 can activate the PI3K-Akt signaling pathway and that p85, the regulatory subunit of PI3K, is one of the major proteins associated with Cbl (15, 26, 46), it is postulated that the activation of Flk-1 and Cbl recruits PI3K to activate its effector Akt. In fact, the products of PI3K activation, such as phosphatidylinositol-3,4,5-trisphosphate and phosphatidylinositol-3,4-bisphosphate, have been reported to bind to the pleckstrin homology domain of Akt and cause its activation (10). Recent reports (39, 42) have indicated that activated Akt forms a physical complex with and activates IKK. Therefore, the present study provides a potential scenario that shear stress and VEGF induce Flk-1-Cbl association to regulate IKK and its subsequent survival signals, with Akt serving as the mediator transmitting the signals from Cbl to IKK.

The present results indicate that the inhibitions of Flk-1 and Cbl abolish VEGF-induced IKK (Fig. 3) but only partially block shear-induced IKK activity (Fig. 2). Our previous study (5) has shown that shear-activated IKK is fully dependent on integrins. Therefore, it is likely that other signaling pathways besides Flk-1/Cbl can also play a role in integrin-mediated shear activation of IKK. In fact, shear stress has been known to activate many signaling molecules downstream of integrins, including FAK and Src (19, 28). These signaling molecules may, together with Flk-1/Cbl, converge on PI3K/Akt to regulate IKK, e.g., activated Src may associate and phosphorylate p85, and therefore regulate PI3K/Akt (47). Figure 4 clearly shows that Akt activation is required for shear- and VEGF-induced IKK activation. Whereas SU-1498 and Cblnm only partially blocked shear-induced IKK activation, Aktnm appears to have completely blocked the induction of IKK by both shear and VEGF. These results suggest that Akt may be the “point of convergence” for these receptor-mediated cascades.

In the present study, the Flk-1 ligand VEGF was shown to be sufficient to induce IKK activation in BAECs (Fig. 1B). This is consistent with reports showing that VEGF induced activation of NF-κB in bovine retinal microvascular ECs, human umbilical vein ECs, and human vascular smooth muscle cells (24, 34, 34, 51). VEGF has also been shown to cause the phosphorylation of IκB-α, a signaling event downstream of the activation of IKK, in HN33 cell lines (20). In contrast, Dikov et al. (9) reported that VEGF had no detectable effect on NF-κB in human dermal microvascular ECs and in fact inhibited TNF-α-induced IKK/NF-κB activation in human hematopoietic progenitor cells. Although there is no clear explanation for these discrepancies, they could be due to differences in cell types and experimental conditions.

In summary, the present results demonstrate that shear stress and VEGF share a common Flk-1/Cbl/Akt signaling pathway in activating IKK. Therefore, signaling events induced by...
mechanical and chemical stimuli may converge on a receptor and regulate downstream signaling pathways with similar molecular mechanisms.

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