Fluid Shear Stress Activation of IκB Kinase Is Integrin-dependent*  

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Vascular endothelial cells (ECs), serving as a barrier between the circulating blood and the vessel wall, are constantly subjected to fluid shear stress due to blood flow. The aim of this study was to determine the role of the recently identified IκB kinases (IKKs) in shear stress activation of NF-κB and to elucidate the upstream signaling mechanism that mediates IKK activation. Our results demonstrate that IKKs in ECs are activated by shear stress in a rapid and transient manner. This IKK activation is followed by IκB degradation and NF-κB translocation into the nucleus. Transfection of plasmids encoding catalytic inactive mutants of IKKs, i.e. hemagglutinin (HA)-IKKα(K44M) and HA-IKKβ(K44A), inhibits shear stress-induced NF-κB translocation. In addition, constructs encoding antisense IKKs, i.e. HA-IKKα(AS) and HA-IKKβ(AS), attenuate shear stress induction of a promoter driven by the κB enhancer element. Preincubation of the EC monolayer with a monoclonal anti-α,β3 integrin antibody (clone LM609) attenuates shear stress induction of IKK. Inhibition of tyrosine kinases by genistein causes a similar down-regulating effect. These results suggest that the integrin-mediated signaling pathway regulates NF-κB through IKKs in ECs in response to shear stress.

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‡‡‡‡‡ The abbreviations used are: ECs, vascular endothelial cells; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor; IKK, IκB kinase; ECM, extracellular matrix; BAECs, bovine aortic endothelial cells; HA, hemagglutinin; mAb, monoclonal antibody; GST, glutathione S-transferase; ROS, reactive oxygen species; MEKK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase.
experiments were performed on BAECs kept on slides for the same duration as many immediately early genes were activated with 10% fetal bovine serum in a humidified 5% CO2 and 95% air incubator at 37 °C. BAECs were cultured on glass slides (38 × 76 mm) to confluence. A silicone gasket was sandwiched between the glass slide and an acrylic plate to create a rectangular flow channel (0.025 cm in height, 2.5 cm in width, and 5.0 cm in length). The BAECs in the 12.5-cm² area was exposed to the applied shear stress, which was generated by circulating the tissue culture medium through a hydrostatic pump connected to the upper and lower reservoirs (30). The pH of the system was kept constant by passing with humidified 95% air and 5% CO2, and the temperature was maintained at 37 °C by keeping the flow system in a temperature-controlled hood. The shear stress, determined by the flow rate perfusing the channel and the channel dimensions, was 12 dynes/cm², which is comparable to the physiological range in the human major arteries and has been found to induce the expression of many immediately early genes in vitro (31, 32). Static control experiments were performed on BAECs kept on slides for the same duration without being exposed to shear stress.

DNA Plasmids and Transient Transfection—The expression plasmids hemagglutinin (HA)-IIKα and HA-IIKβ, which encode HA epitope-tagged IIKα and IIKβ, respectively, and their catalytic inactive mutants, HA-IIKα(K44M) and HA-IIKβ(K44A), were described previously (18). HA-IIKα(AS) and HA-IIKβ(AS) are the antisense forms of HA-IIKα and HA-IIKβ, respectively (18, 19). HIV(LTR)-Luc is a luciferase reporter driven by the human immunodeficiency virus long terminal repeat that contains two binding sites for NF-κB (33). The various DNA plasmids were transfected into BAECs at 80% confluence using the LipofectAMINE method (Life Technologies, Inc.). After incubation for 6 h, the transfected cells were washed with Dulbecco’s modified Eagle’s medium and incubated in fresh Dulbecco’s modified Eagle’s medium to reach confluence. Within 48 h after transfection, the BAEC monolayer was either subjected to shear stress or kept as a static control.

Immunoblotting—BAECs were lysed in a lysis buffer containing 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1% Triton X-100. The lysate was clarified by centrifugation, and the protein concentration of the supernatant was determined using the Bio-Rad protein assay reagent. The protein samples were separated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was blocked with 5% bovine serum albumin, followed by incubation with the primary antibody in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.05% Tween 20 containing 0.1% bovine serum albumin. The bound primary antibodies were detected using horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) and the ECL detection system (Amersham Pharmacia Biotech).

Immunostaining and Fluorescence Microscopy—The translocation of NF-κB was investigated by immunostaining. Confluent monolayers of BAECs were fixed in methanol at –20 °C for 5 min and incubated with 100% goat serum at 4 °C overnight. The specimens were washed three times with phosphate-buffered saline, followed by incubation in phosphate-buffered saline containing 1% bovine serum albumin, 0.2% Triton X-100, and polyclonal anti-NF-κB p65 antibody (1:100, v/v; Santa Cruz Biotechnology, Santa Cruz, CA) and the ECL detection system (Amersham Pharmacia Biotech).

RESULTS

Shear Stress Induces IκBα Degradation and NF-κB Translocation—Khachigian et al. (2) and Lan et al. (4) have previously shown that shear stress increases the binding activity of NF-κB, and we have demonstrated that shear stress increases the transcriptional activity of promoters containing the κB element (5). To test whether the shear stress induction of NF-κB transcriptional activity results from a degradation of IκB proteins and the ensuing NF-κB translocation into the nucleus, BAECs were subjected to a shear stress of 12 dynes/cm² for various time periods. Immunoblotting with polyclonal anti-IκB-α antibody revealed that shear stress caused IκB-α degradation in ECs in a transient manner (Fig. 1A). Compared with static controls, the amount of IκB-α decreased in cells after 10 min of shearing, reached a minimal level at 30 min, and began to increase at 60 min. At 2 h after shearing, cellular IκBα was at the same level as static controls. Immunostaining of the p65 subunit of NF-κB (Fig. 1B) demonstrated that the temporal response of NF-κB translocation was comparable to that of IκB-α degradation. In static ECs, NF-κB was mainly distributed in the cytoplasm, but 15 min of shearing caused some of the nuclei to become anti-NF-κB antibody immunostaining-positive, indicating the translocation of NF-κB from the cytoplasm into the nucleus. At 30 min after shearing, NF-κB was mainly localized in the nucleus, and at 45 min, NF-κB began to reappear in the cytoplasm. Antibody specificity was verified by the absence of NF-κB immunostaining in control experiments in which nonimmune serum was used instead of the primary antibody (i.e. polyclonal anti-NF-κB p65 antibody).

Shear Stress Increases Kinase Activities of IκKs in ECs—Recent findings indicate that IκB proteins are specifically phosphorylated by IκKs, leading to their ubiquitination and degradation by proteasome (18–21, 34). To investigate whether shear stress activates IκKs to up-regulate the NF-κB pathway in ECs, BAECs were transfected with plasmids encoding HA-IκKα and HA-IκKβ and subjected to shear stress experiments. Using GST-IκBα(1–54) as the substrate, immunocomplex kinase activity assay showed that IκK activity associated with HA-IκKα and HA-IκKβ was increased by shear stress, similar to the IκK activity induced by the treatment of TNF-α (Fig. 2). The shear stress activation of IκKs occurred as early as 5 min, peaked at 30 min, and returned to the basal level 2 h after shearing. Densitometric analysis showed that...
NF-κB translocation in ECs.

Fig. 1. Shear stress induces IκB-α degradation and NF-κB translocation in ECs. Confluent BAEC monolayers were kept as static controls (time 0) or subjected to a shear stress of 12 dynes/cm² for various time periods as indicated. In A, cell lysates from various samples were immunoblotted with polyclonal anti-IκB-α antibody. The transient degradation of IκB-α is demonstrated by the decreased IκB-α immunoblotting in cells sheared for 30 min and 1 h. Bar graphs, representing the mean ± S.D. from three separate experiments, show the level of IκB-α in the various samples relative to that in the static controls. In B, cells were sheared for various periods of time and fixed, and immunostaining was performed with polyclonal anti-NF-κB p65 antibody, followed by fluorescein-conjugated goat anti-rabbit IgG. The subcellular distribution of NF-κB was observed under a fluorescence microscope.

NF-κB p65 antibodies was performed to detect NF-κB translocation in the plasmid-transfected cells. Whereas anti-HA antibody identified the transfected cells, anti-NF-κB p65 antibody revealed the distribution of NF-κB in these transfected cells. As shown in Fig. 3, shear stress induced the translocation of NF-κB from the cytoplasm into the nucleus in the non-transfected cells. In contrast, the transfection of HA-IKKα(K44M) and HA-IKKβ(K44A) blocked the NF-κB translocation induced by shear stress. In parallel control experiments, the transfection of wild-type HA-IKKα and HA-IKKβ did not affect the shear-induced translocation of NF-κB (data not shown).

Antisense IKKs Attenuate Shear Stress Induction of Luciferase Driven by the κB Element—HA-IKKα(AS) and HA-IKKβ(AS) encode the antisense forms of HA-IKKα and HA-IKKβ, respectively (18, 19). HIV(LTR)-Luc is a shear-inducible construct, with its induction mediated by the κB element (5). To further confirm that the shear stress-induced NF-κB transcriptional activity is regulated by the IKKs, HIV(LTR)-Luc was cotransfected with HA-IKKα(AS) or HA-IKKβ(AS). As shown in Fig. 4, in BAECs cotransfected with HIV(LTR)-Luc and the pB-Rα3 parental vector, shear stress caused an increase in luciferase activity to 3.2-fold of the static controls. However, cotransfection with HA-IKKα(AS) or HA-IKKβ(AS) or a combination of both abolished this shear stress induction of luciferase activity.

Integrins Are Involved in Shear Stress Activation of IKK—The data presented in Figs. 1–4 show that application of shear stress to ECs activates NF-κB through the induction of IKKs. An important question is what are the upstream molecules that mediate the mechanotransduction to activate IKKs. The activation of mitogen-activated protein kinases by shear stress is similar to that induced by attachment of cells to the ECM or incubation of cells with beads coated with integrin ligands or...
anti-integrin antibodies (see Ref. 29 for review). Thus, we investigated whether integrins regulate the shear stress activation of IKKs in ECs. Confluent monolayers of BAECs transfected with HA-IKKα and HA-IKKβ were preincubated for 3 h with LM609, a mAb against the abundant endothelial αvβ3 integrin. With such an incubation, the applied antibody has been shown to gain access to the abluminal side of the cells (35). A shear stress of 12 dynes/cm² was applied to these LM609-incubated cells for 30 min, followed by immunocomplex kinase assay for HA-IKKα and HA-IKKβ. As shown in Fig. 5, preincubation of BAECs with LM609 attenuated shear stress activation of these HA-IKKs, as indicated by the decreased phosphorylation of GST-IκBα-(1–54) compared with cells that had been exposed to mouse IgG. These results suggest that αvβ3 integrin is involved in the mechanotransduction that mediates the shear stress activation of IKK pathways.

Protein-tyrosine kinases in the focal adhesions are commonly involved in integrin-mediated signal transduction (see Ref. 36 for review). To examine the roles of protein-tyrosine kinases in shear stress activation of IKKs, confluent monolayers of BAECs transfected with HA-IKKα and HA-IKKβ were pretreated with a protein-tyrosine kinase inhibitor (genistein), followed by shear stress experiments and immunocomplex kinase assay. As shown in Fig. 5, pretreatment of BAECs with genistein attenuated shear stress activation of HA-IKKs. Integrin-mediated signal transduction is usually investigated in cells adhered to the ECM. Many of these signaling events are similar to those involved in cellular responses to

FIG. 3. Catalytic inactive mutants of IKKs block shear-induced NF-κB translocation. BAECs were transfected with HA-IKKα(K44M) or HA-IKKβ(K44A), the respective catalytic inactive mutants of HA-IKKα and HA-IKKβ. These transfected BAECs were kept as static controls or subjected to a shear stress of 12 dynes/cm² for 30 min. After fixation, double immunostaining was performed with polyclonal anti-NF-κB antibody and anti-HA mAb. The primary antibodies were conjugated to fluorescein-conjugated goat anti-rabbit IgG and rhodamine-conjugated goat anti-mouse IgG, respectively. The transfected cells were identified by the detection of rhodamine staining. The subcellular localization of NF-κB in these transfected cells was visualized by the distribution of fluorescein.

FIG. 4. Antisense forms of IKKs attenuate the transcriptional activation of NF-κB in response to shear stress. BAECs were transfected with HIV(LTR)-Luc, pSV-β-galactosidase, and antisense forms of IKKs, viz. HA-IKKα(AS) and HA-IKKβ(AS). In parallel experiments, cells were transfected with HIV(LTR)-Luc, pSV-β-galactosidase, and the pSRα3 parental vector. The transfected cells were kept as static controls or subjected to a shear stress of 12 dynes/cm² for 8 h. Cells were then lysed for luciferase and β-galactosidase activities assays. The normalized luciferase activities are the luminometer readings of the luciferase activity corrected for transfection efficiency based on the β-galactosidase activity. The results represent the mean ± S.D. from three separate experiments.

FIG. 5. Anti-αvβ3 integrin antibody and genistein attenuate shear stress activation of IKKs. BAECs were transfected with HA-IKKα and HA-IKKβ were treated with mouse IgG (10 μg/ml), anti-αvβ3 integrin mAb (clone LM609; 10 μg/ml), or genistein (100 μM) for 3 h. The treated cells were then subjected to a shear stress of 12 dynes/cm² for 30 min or a static incubation for the same length of time. The procedures for immunocomplex kinase assays were the same as those described in the legend to Fig. 2. The bands indicated by the arrow represent phosphorylated GST-IκBα-(1–54). Bar graphs, representing the mean ± S.D. from three separate experiments, show the kinase activities of the various samples relative to those in the untreated static controls. Shown in the lower panel is immunoblotting with anti-HA mAb, indicating that comparable amounts of HA-IKKα and HA-IKKβ were expressed in the various experiments.
shear stress (see Ref. 29 for review). To further confirm that IKK can be activated by integrin-mediated signal transduction, HA-IKKα and HA-IKKβ-transfected BAECs in suspension were allowed to adhere to a fibronectin-coated surface and then subjected to immunocomplex kinase assay. As shown in Fig. 6, the temporal response of these HA-IKKs during EC adhesion was similar to that in ECs exposed to shear stress. The peak activity occurred at 15 min; by 1 h after cell attachment, the activity was at a basal level similar to that in the suspension.

In addition to fibronectin, the activation of IKKs was also observed in cells adhered to vitronectin and collagen, but not to poly-L-lysine (data not shown).

**DISCUSSION**

Reperfusion injury results in many responses, including the release of reactive oxygen species (ROS) and the expression of genes that are mediated by the transcription factor NF-κB (see Ref. 37 for review). The sudden application of shear stress to ECs cultured in a flow channel mimics the reperfusion process. Using such an in vitro model, we (5) and others (2–4) have previously shown that shear stress increases the transcriptional activity of NF-κB. In this investigation on the upstream signal transduction pathway leading to the shear stress induction of NF-κB in ECs, we found that shear stress activates an IKK/NF-κB pathway and that this is at least in part mediated by integrins such as α3β1 integrin.

The recently identified IKKα and IKKβ constitute two components of the IKK complex that phosphorylates the serine residues in IκB (18–21, 34). Specific serine phosphorylation, such as at Ser-32 and Ser-36 of IκBα, leads to the ubiquitination and degradation of IκB (18, 19, 21). The temporal responses of ECs to shear stress in terms of IKK activation, IκB degradation, NF-κB translocation, and NF-κB-mediated transcriptional activation reveal the following events. Under static condition, NF-κB in ECs is sequestered in the cytoplasm by the binding of IκB. Shear stress activates IKKs, which phosphorylate IκB to lead to its degradation. As a consequence, the NF-κB released from the NF-κB/IκB complex translocates into the nucleus to activate its target genes.

In addition to the augmented expression of the NF-κB-mediated genes, the functional consequences of shear stress activation of the IKK/NF-κB pathway in ECs may also include the modulation of cell survival, apoptosis, and motility. During the preparation of this manuscript, Scatena et al. (38) reported that adhesion of ECs to osteopontin activates NF-κB and thus rescues cells from apoptosis induced by serum deprivation. It was further suggested that the NF-κB activation through α3β1 integrin mediates this EC survival since anti-β1 integrin mAb F11 blocks NF-κB activity and induces EC apoptosis. The activation of NF-κB by TNF, ionizing radiation, or the ras proto-oncogene was found to protect cells from apoptosis (39–41). Inhibition of NF-κB nuclear translocation enhances apoptotic killing by these reagents, but not by apoptotic stimuli that do not activate NF-κB. Recent studies showed that shear stress protects ECs from apoptosis: TNF- or H2O2-induced EC apoptosis is inhibited by preconditioning the EC monolayer with a shear stress of 15 dynes/cm² (42, 43). Although the anti-apoptotic effects of shear stress have been linked to the production of nitric oxide (42), it would be interesting to investigate whether the augmented IKK/NF-κB pathway is also involved. α3β1 integrin, in conjunction with activated protein kinase C, promotes the migration of FG carcinoma cells on vitronectin (44). An oligonucleotide-containing κB element, when introduced into FG carcinoma cells, inhibited the NF-κB-mediated cell motility (45). In a disturbed flow field, there is a net EC migration directed away from the region of the high shear stress gradient (46). This organized migration pattern under disturbed flow conditions is accompanied by a >2-fold increase in cell motility. Thus, shear stress activation of the IKK/NF-κB pathway and of protein kinase C (47) may regulate EC motility, which would be important for the morphological remodeling of ECs.

Many extracellular stimuli activate NF-κB, presumably acting through IKK due to its specificity in phosphorylating IκB (19, 20). However, the upstream signaling events activated by the various stimuli that converge at IKKs have not yet been clearly established. There is increasing evidence to indicate that integrins are important in mechanotransduction in cells in response to mechanical stimuli (see Ref. 29 for review). Indeed, the results in Fig. 5 suggest that integrins are directly involved in the shear stress activation of IKKs in ECs. The integrin-mediated signaling during cell adhesion to the ECM often results in an increase in the activity of tyrosine kinases in the focal adhesion sites, including focal adhesion kinase and Src family proteins (see Ref. 28 for review). Previous studies have shown that shear stress activates focal adhesion kinase and Src family kinases (35, 48, 49). The inhibition of IKKs by genistein (Fig. 5) and the activation of IKKs when ECs attached to the ECM (Fig. 6) further confirmed that integrins are upstream molecules modulating IKKs. The observations that the isolated IKK complex from unstimulated cells can be activated in vitro by MEKK and that overexpression of MEKK in cells leads to the phosphorylation of IκBα (50) indicate that the IKK/NF-κB pathway can be activated by MEKK. We have previously shown that Ras and MEKK modulate shear stress activation of c-Jun N-terminal kinase since dominant-negative mutants of Ras and MEKK block c-Jun N-terminal kinase activation in ECs (51). Taken together, the previous studies suggest that integrins are activated by shear stress. As a consequence, the Ras/MEKK pathway is activated in a focal adhesion kinase- and c-Src-dependent manner, which in turn causes the activa-
Shear Stress Activation of IKK

In addition to integrins, other upstream signaling molecules such as the TNF receptor (TNFR), CD95 (Fas/Apo-1), and ROS may also be involved in the shear stress activation of IKKs. In the TNF induction of NF-κB, the signal transduces through the TNFR, TNFR-associated factor 2, and NF-κB-inducing kinase (52, 53). NF-κB-inducing kinase is an IKK kinase that has been shown to activate IKKs, possibly through a direct interaction (21, 34). In addition to the TNFR, NF-κB-inducing kinase is also involved in CD95-mediated NF-κB activation (52). We have found that shear stress causes the clustering of membrane-associated proteins such as Flk-1, which is a receptor for the vascular endothelium growth factor. It is likely that shear-generated ROS also modulates the activation of the shear stress induction of ROS is still unknown, it is likely that the shear-generated ROS also modulates the activation of IKKs.

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