

Fluid Shear Stress Activation of I κ B Kinase Is Integrin-dependent*

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Vascular endothelial cells (ECs), forming a boundary 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 I κ B kinases (IKKs) in shear stress activation of NF- κ B 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 I κ B degradation and NF- κ B translocation into the nucleus. Transfection of plasmids encoding catalytic inactive mutants of IKKs, *i.e.* hemagglutinin (HA)-IKK α (K44M) and HA-IKK β (K44A), inhibits shear stress-induced NF- κ B translocation. In addition, constructs encoding antisense IKKs, *i.e.* HA-IKK α (AS) and HA-IKK β (AS), attenuate shear stress induction of a promoter driven by the κ B enhancer element. Preincubation of the EC monolayer with a monoclonal anti- $\alpha_v\beta_3$ 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- κ B through IKKs in ECs in response to shear stress.

Vascular endothelial cells (ECs),¹ serving as a barrier between the circulating blood and the vessel wall, are constantly exposed to fluid shear stress. The focal nature of atherosclerotic lesions in the arterial tree demonstrates the critical role of flow conditions in atherogenesis. *In vitro* experiments using flow channels with cultured ECs have shown that shear stress activates the platelet derived-growth factor gene (1), a potent mitogen for vascular smooth muscle cells. The shear stress activation of the platelet derived-growth factor gene is through the action of the transcription factor NF- κ B on the shear stress-responsive element GAGACC (2, 3). In addition, electrophoresis mobility shift assay showed that nuclear extracts isolated from ECs exposed to shear stress increase their binding to

oligonucleotides containing the κ B enhancer element (4), and a luciferase reporter driven by the κ B enhancer element was shown to be shear-inducible (5). However, the signal transduction pathway leading to the activation of NF- κ B in ECs in response to shear stress is still unclear.

The transcription factor NF- κ B was first identified as a protein that binds to a specific DNA site in the intronic enhancer of the immunoglobulin κ light chain gene (6). It is composed of homo- or heterodimers of members of the Rel family of transcription factors that control the expression of numerous genes involved in the immune and inflammatory responses, cell adhesion, and growth control (see Refs. 7 and 8 for review). NF- κ B can be activated by many types of extracellular stimuli, including tumor necrosis factor (TNF), interleukin-1, bacterial endotoxin lipopolysaccharide, viral infection, viral proteins, antigen receptor cross-linking of T and B cells, calcium ionophores, phorbol esters, UV radiation, free radicals, hypoxia, etc. (see Ref. 9 for review). In almost all cell types, NF- κ B is sequestered in the cytoplasm through tight association with the inhibitory I κ B proteins, including I κ B- α and I κ B- β . Activation of NF- κ B by a variety of stimuli is dependent upon the phosphorylation and subsequent degradation of the I κ B proteins; this allows the translocation of NF- κ B into the nucleus to activate various target genes. Phosphorylation of I κ B proteins occurs at specific residues, Ser-32 and Ser-36 of I κ B- α and Ser-19 and Ser-23 of I κ B- β (10–13). Following phosphorylation, I κ B proteins are ubiquitinated and then degraded by a proteasome-dependent pathway (10–17). The I κ B kinase (IKK) complex was recently purified and is composed of several subunits (18–21). Two of the subunits, 85 and 87 kDa in size, were termed IKK α and IKK β , respectively. These two proteins (with 52% homology) contain an N-terminal catalytic kinase domain and several putative protein interaction motifs, including a leucine zipper and a helix-loop-helix domain at their C termini (18, 19).

Integrins are a family of >20 different transmembrane heterodimers composed of α - and β -subunits that are associated noncovalently. All integrins consist of a large extracellular domain, a transmembrane region, and a relatively short cytoplasmic region. The extracellular domain typically binds to an Arg-Gly-Asp (RGD) sequence that is present in various extracellular matrix (ECM) ligands, *e.g.* fibronectin, vitronectin, and collagen. The cytoplasmic domain, generally consisting of 20–70 amino acids, interacts with cytoskeletal proteins, *e.g.* actin filaments, and kinases in the focal adhesion sites, *e.g.* focal adhesion kinase and c-Src (see Refs. 22 and 23 for review). $\alpha_4\beta_1$ integrin is involved in the NF- κ B-mediated gene expression in leukocytes (24–26). The RGD motif of the ECM appears to be involved in the NF- κ B activation during cell-cell interaction (27). There is ample evidence demonstrating that integrin-mediated signaling regulates the mitogen-activated protein kinase pathways including extracellular signal-regulated kinase and c-Jun N-terminal kinase (see Ref. 28 for review). We have proposed that integrins in ECs can serve as mechanosensors

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¹ The abbreviations used are: ECs, vascular endothelial cells; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor; IKK, I κ B 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.

(29). In this study, we present evidence that integrins such as $\alpha_v\beta_3$ transduce mechanical stimuli into biochemical signals to activate NF- κ B through IKKs in ECs in response to shear stress.

EXPERIMENTAL PROCEDURES

Cell Cultures and Shear Stress Experiment—Bovine aortic endothelial cells (BAECs) prior to passage 10 were used in all experiments. The cells were maintained in Dulbecco's modified Eagle's medium supplemented 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 gassing 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)-IKK α and HA-IKK β , which encode HA epitope-tagged IKK α , and IKK β , respectively, and their catalytic inactive mutants, HA-IKK α (K44M) and HA-IKK β (K44A), were described previously (18). HA-IKK α (AS) and HA-IKK β (AS) are the antisense forms of HA-IKK α and HA-IKK β , 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- κ B (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 centrifuged, 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- κ B 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- κ B p65 antibody (1:100, v/v; Santa Cruz Biotechnology) for 2 h at 37 °C. After being washed three times with phosphate-buffered saline containing 0.2% Triton X-100, the specimens were incubated with fluorescein-conjugated anti-rabbit IgG (1:200, v/v; Sigma) for 2 h at room temperature. In the inhibition experiments, BAECs were transfected with HA-IKK α (K44M) or HA-IKK β (K44A). An anti-HA mAb (Boehringer Mannheim) and anti-NF- κ B p65 antibody were used in double immunostaining (18). These antibodies were detected using rhodamine-conjugated anti-mouse IgG and fluorescein-conjugated anti-rabbit IgG, respectively. The immunostaining was observed under an epifluorescence microscope. Fluorescein was excited at a wavelength of 488 nm and detected between 506 and 538 nm, whereas rhodamine was excited at 568 nm and detected between 589 and 621 nm.

IKK Immunocomplex Kinase Activity Assay—BAECs transfected with HA-IKK α and HA-IKK β were lysed in a lysis buffer containing 20 mM Tris-HCl, pH 7.6, 0.3 M NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton

X-100, 2 mM dithiothreitol, 20 mM β -glycerophosphate, 1 mM Na₃VO₄, 10 μ g/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 10 μ g/ml aprotinin. HA-IKK α and HA-IKK β were immunoprecipitated from the cell lysate using protein A-Sepharose beads and anti-HA mAb. After centrifugation, the pelleted immunocomplex was washed with the lysis buffer, followed by washing first with a lysis buffer containing 1 M urea and then with an IKK assay buffer containing 20 mM Hepes, pH 7.4, 20 mM MgCl₂, 20 mM dithiothreitol, 20 mM β -glycerophosphate, 0.1 mM Na₃VO₄, and 10 μ g/ml aprotinin. The immunocomplex was then resuspended in 20 μ l of the kinase buffer containing 5 μ Ci of [γ -³²P]ATP, 20 μ M ATP, and 1 μ g of glutathione S-transferase (GST)-I κ B- α (1-54). After incubation at 30 °C for 30 min, the kinase reaction mixture was resolved on a 10% SDS-polyacrylamide gel, and phosphorylated GST-I κ B- α was detected by autoradiography (18).

Luciferase Activity Assays—HIV(LTR)-Luc, HA-IKK α (AS), and HA-IKK β (AS) were cotransfected into BAECs for the luciferase induction assay. The pSV- β -galactosidase plasmid, which contains a β -galactosidase gene driven by the SV40 promoter and enhancer, was also cotransfected to monitor the transfection efficiency. To release the reporter luciferase and β -galactosidase, the cells were lysed with a lysis buffer containing 1% Triton X-100. ATP and luciferin were then added to the lysate in a luminometer for measuring the total light output. The level of β -galactosidase was assayed by adding the substrate *o*-nitrophenyl- β -galactopyranoside to the cell lysate and incubating at 37 °C for 1 h. The reaction was then quenched by the addition of Na₂CO₃, and the absorbance at 410 nm was recorded. The luciferase activity was normalized with that of β -galactosidase.

RESULTS

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

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

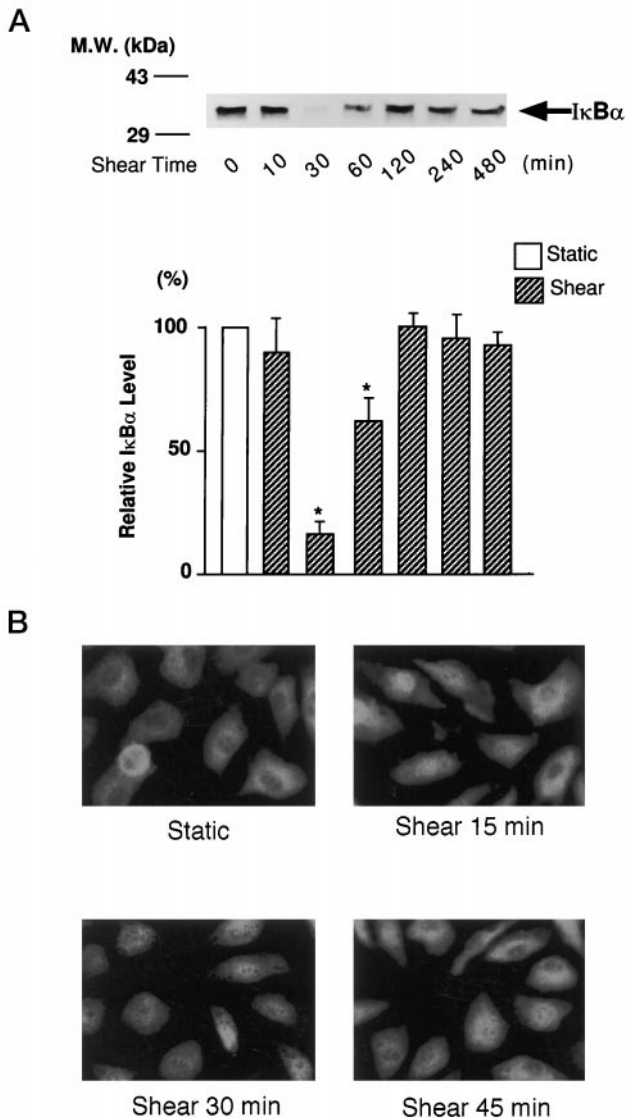


FIG. 1. Shear stress induces IκB-α degradation and NF-κB translocation in ECs. Confluent BAEC monolayers were kept as static controls (time 0) or subjected to a shear stress of 12 dynes/cm² for various time periods as indicated. In *A*, cell lysates from various samples were immunoblotted with polyclonal 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.

the peak activity was three times the static controls. The temporal change of IKK activity is similar to that of IκB degradation (Fig. 1A), suggesting that shear stress activates IKKs, which phosphorylate IκB proteins to cause their degradation.

Catalytic Inactive Mutants of IKKs Inhibit Shear Stress-induced NF-κB Translocation—To test whether inhibition of IKKs abolishes shear stress-induced NF-κB translocation, BAECs were transfected with plasmids encoding HA-IKKα(K44M) and HA-IKKβ(K44A), the respective mutants of catalytic inactive HA-IKKα and HA-IKKβ (18, 19). In parallel control experiments, cells were transfected with wild-type HA-IKKα and HA-IKKβ. The transfected BAECs were subjected to a shear stress of 12 dynes/cm² for 30 min or kept as static controls. Double immunostaining using anti-HA and anti-

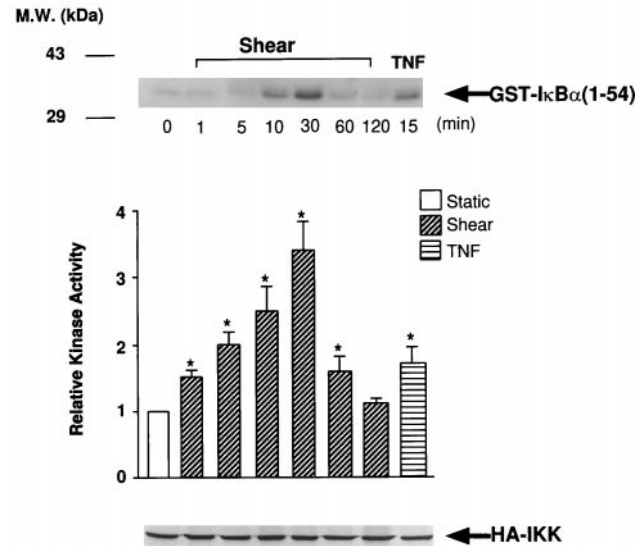


FIG. 2. Shear stress activates IKKs in ECs. BAECs were transfected with plasmids encoding the HA epitope-tagged IKKs (*i.e.* HA-IKKα and HA-IKKβ). The transfected cells were kept as static controls (time 0), subjected to a shear stress of 12 dynes/cm² for various time periods as indicated, or treated with TNF-α (20 ng/ml) for 15 min. HA-IKKα and HA-IKKβ were immunoprecipitated with anti-HA mAb for kinase activity assay using GST-IκB-α(1-54) and [³²P]ATP as substrates. The bands indicated by the *arrow* represent phosphorylated GST-IκB-α(1-54) after SDS-polyacrylamide gel electrophoresis and autoradiography. *Bar graphs*, representing the mean ± S.D. from three separate experiments, show the kinase activity of the various samples relative to that in the 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.

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-increased 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 pSRα3 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

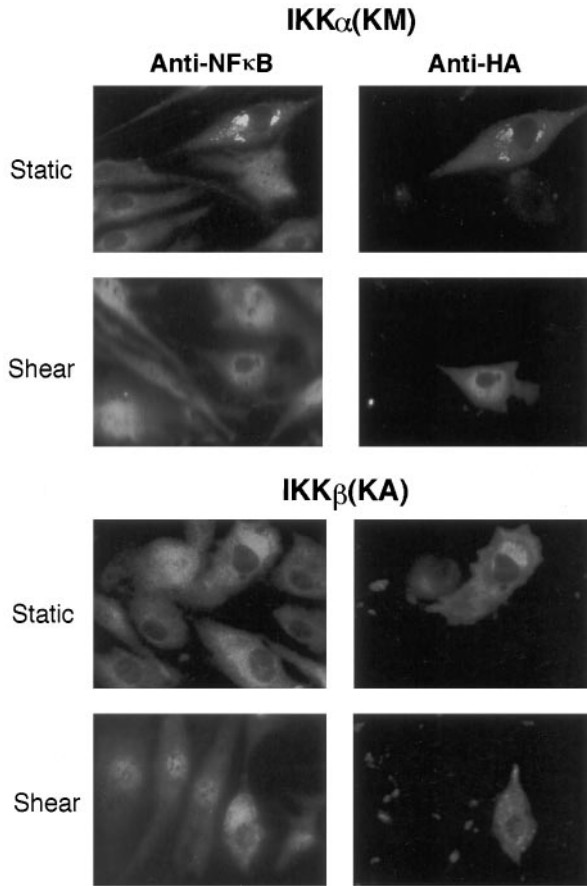


FIG. 3. Catalytic inactive mutants of IKKs block shear-induced NF- κ B translocation. BAECs were transfected with HA-IKK α (K44M) or HA-IKK β (K44A), the respective catalytic inactive mutants of HA-IKK α and HA-IKK β . These transfected BAECs were kept as static controls or subjected to a shear stress of 12 dynes/cm² for 30 min. After fixation, double immunostaining was performed with polyclonal anti-NF- κ B 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- κ B in these transfected cells was visualized by the distribution of fluorescein.

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-IKK α and HA-IKK β were preincubated for 3 h with LM609, a mAb against the abundant endothelial $\alpha_v\beta_3$ 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-IKK α and HA-IKK β . 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-I κ B- α (1-54) compared with cells that had been exposed to mouse IgG. These results suggest that $\alpha_v\beta_3$ 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-IKK α and HA-IKK β 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

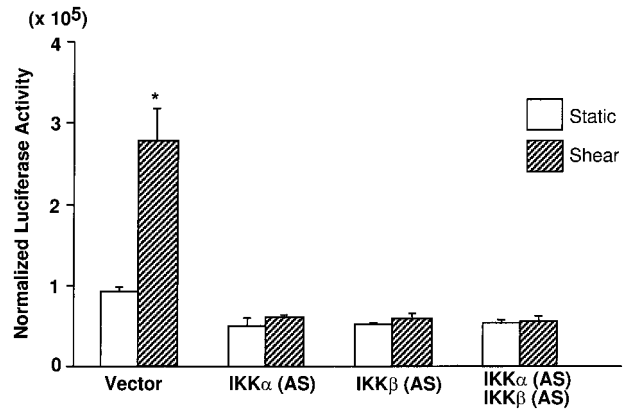


FIG. 4. Antisense forms of IKKs attenuate the transcriptional activation of NF- κ B in response to shear stress. BAECs were transfected with HIV(LTR)-Luc, pSV- β -galactosidase, and antisense forms of IKKs, *viz.* HA-IKK α (AS) and HA-IKK β (AS). In parallel experiments, cells were transfected with HIV(LTR)-Luc, pSV- β -galactosidase, and the pSR α 3 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 \pm S.D. from three separate experiments.

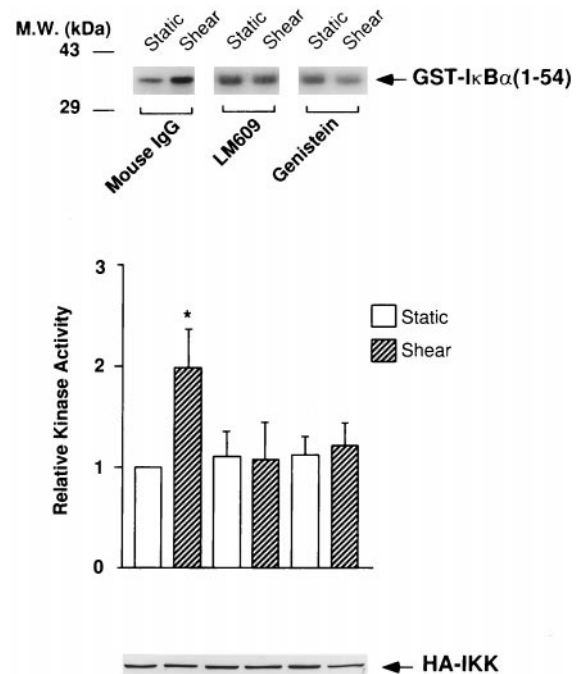


FIG. 5. Anti- $\alpha_v\beta_3$ integrin antibody and genistein attenuate shear stress activation of IKKs. BAECs transfected with HA-IKK α and HA-IKK β were treated with mouse IgG (10 μ g/ml), anti- $\alpha_v\beta_3$ integrin mAb (clone LM609; 10 μ g/ml), or genistein (100 μ M) for 3 h. The treated cells 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-I κ B- α (1-54). Bar graphs, representing the mean \pm 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-IKK α and HA-IKK β were expressed in the various experiments.

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

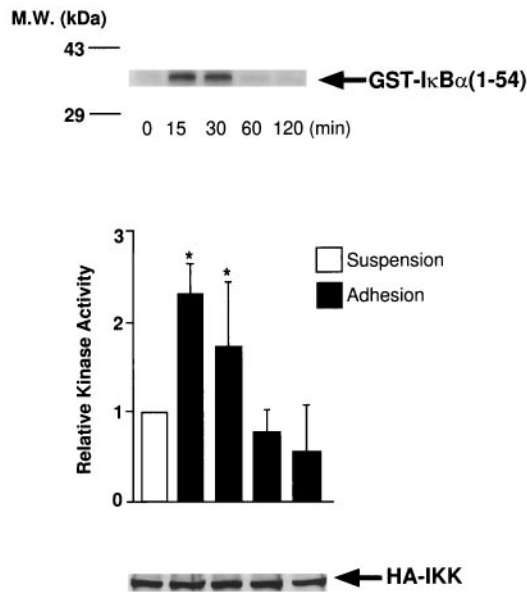


FIG. 6. Adhesion of ECs to fibronectin activates IKKs. BAECs transfected with HA-IKK α and HA-IKK β were trypsinized and allowed to adhere to a fibronectin-coated surface for various time periods as indicated. Cells were then lysed for IKK immunocomplex kinase assay. The bands indicated by the arrow represent phosphorylated GST-I κ B α (1-54). Bar graphs, representing the mean \pm S.D. from three separate experiments, show kinase activities of the various samples relative to those in suspension controls (time 0). Shown in the lower panel is immunoblotting with anti-HA mAb, indicating that comparable amounts of HA-IKK α and HA-IKK β 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-IKK α - and HA-IKK β -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- κ B (see Ref. 37 for review). The sudden application of shear stress to ECs cultured in a flow channel mimics the reperfusion process in the vessels. Using such an *in vitro* model, we (5) and others (2-4) have previously shown that shear stress increases the transcriptional activity of NF- κ B. In this investigation on the upstream signal transduction pathway leading to the shear stress induction of NF- κ B in ECs, we found that shear stress activates an IKK/NF- κ B pathway and that this is at least in part mediated by integrins such as $\alpha_v\beta_3$ integrin.

The recently identified IKK α and IKK β constitute two components of the IKK complex that phosphorylates the serine residues in I κ B (18-21, 34). Specific serine phosphorylation, such as at Ser-32 and Ser-36 of I κ B- α , leads to the ubiquitination and degradation of I κ B (18, 19, 21). The temporal responses of ECs to shear stress in terms of IKK activation, I κ B degradation, NF- κ B translocation, and NF- κ B-mediated transcriptional activation reveal the following events. Under static condition, NF- κ B in ECs is sequestered in the cytoplasm by the

binding of I κ B. Shear stress activates IKKs, which phosphorylate I κ B to lead to its degradation. As a consequence, the NF- κ B released from the NF- κ B-I κ B complex translocates into the nucleus to activate its target genes.

In addition to the augmented expression of the NF- κ B-mediated genes, the functional consequences of shear stress activation of the IKK/NF- κ B 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- κ B and thus rescues cells from apoptosis induced by serum deprivation. It was further suggested that the NF- κ B activation through $\alpha_v\beta_3$ integrin mediates this EC survival since anti- β_3 integrin mAb F11 blocks NF- κ B activity and induces EC apoptosis. The activation of NF- κ B by TNF, ionizing radiation, or the *ras* proto-oncogene was found to protect cells from apoptosis (39-41). Inhibition of NF- κ B nuclear translocation enhances apoptotic killing by these reagents, but not by apoptotic stimuli that do not activate NF- κ B. 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- κ B pathway is also involved. $\alpha_v\beta_3$ integrin, in conjunction with activated protein kinase C, promotes the migration of FG carcinoma cells on vitronectin (44). An oligonucleotide-containing κ B element, when introduced into FG carcinoma cells, inhibited the NF- κ B-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- κ B 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- κ B, presumably acting through IKK due to its specificity in phosphorylating I κ B (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 I κ B- α (50) indicate that the IKK/NF- κ B 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-

tion of IKKs.

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- κ B, the signal transduces through the TNFR, TNFR-associated factor 2, and NF- κ B-inducing kinase (52, 53). NF- κ B-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- κ B-inducing kinase is also involved in CD95-mediated NF- κ B 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.² It is likely that shear stress activates the TNFR and CD95 through a similar clustering-dependent mechanism and that this in turn leads to the activation of the NF- κ B-inducing kinase/IKK pathway. The NF- κ B/I κ B pathway is potentiated by ROS, but attenuated by antioxidants (see Ref. 37 for review). Application of shear stress to ECs induces the production of ROS (54). Although the nature 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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² K.-D. Chen and J. Y.-J. Shyy, unpublished results.