Mechanotransduction by integrin is essential for IL-6 secretion from endothelial cells in response to uniaxial continuous stretch

Akitoshi Sasamoto,1,2 Masato Nagino,1 Satoshi Kobayashi,1 Keiji Naruse,2,3 Yuji Nimura,1 and Masahiro Sokabe2,3,4

1Division of Surgical Oncology, Department of Surgery, and 2Department of Physiology, Nagoya University Graduate School of Medicine; and 3Cell Mechanosensing Project, International Cooperative Research Project, Japan Science and Technology Agency, Nagoya; and 4Department of Molecular Physiology, National Institute for Physiological Sciences, Okazaki, Japan

Submitted 8 July 2004; accepted in final form 17 December 2004

Sasamoto, Akitoshi, Masato Nagino, Satoshi Kobayashi, Keiji Naruse, Yuji Nimura, and Masahiro Sokabe. Mechanotransduction by integrin is essential for IL-6 secretion from endothelial cells in response to uniaxial continuous stretch. Am J Physiol Cell Physiol 288: C1012–C1022, 2005. First published December 22, 2004; doi:10.1152/ajpcell.00314.2004.—We previously reported that uniaxial continuous stretch in human umbilical vein endothelial cells (HUVECs) induced interleukin-6 (IL-6) secretion via IκB kinase (IKK)/nuclear factor-κB (NF-κB) activation. The aim of the present study was to clarify the upstream signaling mechanism responsible for this phenomenon. Stretch-induced IKK activation and IL-6 secretion were inhibited by application of αβ1 integrin-inhibitory peptide (GRGDNP), phosphatidylinositol 3-kinase inhibitor (LY-294002), phospholipase C-γ inhibitor (U-73122), or protein kinase C inhibitor (H7). Although depletion of intra- or extracellular Ca2+ pool using thapsigargin (TG) or EGTA, respectively, showed little effect, a TG-EGTA mixture significantly inhibited stretch-induced IKK activation and IL-6 secretion. An increase in the intracellular Ca2+ concentration ([Ca2+]i) upon continuous stretch was observed even in the presence of TG, EGTA, or GRGDNP, but not in a solution containing the TG-EGTA mixture, indicating that both integrin activation and [Ca2+]i rise are crucial factors for stretch-induced IKK activation and after IL-6 secretion in HUVECs. Furthermore, while PKC activity was inhibited by the TG-EGTA mixture, GRGDNP, LY-294002, or U-73122, PLC-γ activity was retarded by GRGDNP or LY-294002. These results indicate that continuous stretch-induced IL-6 secretion in HUVECs depends on outside-in signaling via integrins followed by a PKC-PLC-γ-IKK/NF-κB signaling cascade. Another crucial factor, [Ca2+]i increase, may at least be required to activate PKC needed for NF-κB activation.

nuclar factor-κB; phosphatidylinositol 3-kinase; phospholipase C-γ; protein kinase C; intracellular Ca2+ concentration

VASCULAR ENDOTHELIAL CELLS (ECs) that line the vascular system are subjected to three major hemodynamic forces, shear stress, transmural pressure, and mechanical stretch. Major hemodynamic forces loading ECs are shear stress by blood flow and circumferential (uniaxial) cyclic tension by vessel expansion. On the other hand, hemodynamic changes associated with clinical morbidity often cause an abrupt and sustained dilatation of vessels, leading to uniaxial continuous mechanical stretch on ECs (44). Blood congestion due to acute heart failure or to acute obstruction of vessels produces a rapid and sustained stretch load on ECs followed by secretions of a variety of chemical mediators, which in turn modify the morbidity conditions (12, 26, 29). We previously reported that an ~150% uniaxial sustained stretch in sinusoidal ECs after portal vein embolization might be a trigger for liver regeneration via interleukin-6 (IL-6) secretion (21). Furthermore, we demonstrated that IL-6 secretion from ECs in response to sustained stretch is mediated by a sequential activation of IκB kinase (IKK) and nuclear factor (NF)-κB (22). However, the signal transduction pathway leading to IKK activation after sustained stretch in ECs remained to be elucidated.

Experimentally applied continuous stretch to ECs consists of a single immediate transient load followed by a sustained tonic load. We previously showed that the sustained tonic component of uniaxial continuous stretch is more important than the single immediate transient component in the stretch-induced IL-6 secretion from ECs (22). Therefore, mechanotransduction mechanisms activated by tonic force during sustained stretch seem to be crucial to understanding the signaling mechanism causing IL-6 secretion. Integrins, which comprise a major family of transmembrane receptors that mediate cell adhesion to extracellular matrices (ECMs), are one of the potential candidate molecules to transduce tonic force into intracellular biochemical signals (4, 23, 25). Bhullar et al. (4) reported that the integrin-mediated signaling pathway regulates NF-κB through the activation of IKK in bovine aortic endothelial cells in response to shear stress, although its detailed signaling events have not been clarified yet.

Several studies of mechanical force-evoked integrin signaling have indicated that integrin activates NF-κB in a phosphatidylinositol 3-kinase (PI3-kinase)-dependent manner (9, 37). PI3-kinase is a lipid kinase that phosphorylates phosphoinositides at the 3′-position of the inositol ring. The principal products of PI3-kinase, PI(3,4)P2 and PI(3,4,5)P3, act as second messengers that activate the serine/threonine kinase Akt/ PKB (15). Alternatively, the binding of the produced PI(3,4,5)P3 to the src homology domain 2 (SH2) or to the pleckstrin homology domain (PH) of phospholipase C-γ (PLC-γ) leads to the activation of PLC-γ (2, 11, 35). PLC-γ plays an important role in the regulation of various intracellular signaling mechanisms. Activated PLC-γ hydrolyzes PI(4,5)P2 to inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG), which contribute to an increase in intracellular Ca2+ concen-
tration ([Ca^{2+}]) and activation of protein kinase C (PKC), respectively (16, 33). PKC is involved in stretch-induced reactive oxygen species (ROS) production, such as H_{2}O_{2}, lipid peroxides, and O_{2} \ (5, 7, 14, 27), followed by activation of NF-κB (13, 49). We also previously reported that an antioxidant inhibits the IL-6 production by suppressing NF-κB (22).

A number of studies have shown that Ca^{2+} is essential for production of ROS (1, 3, 34). Elevations of [Ca^{2+}] is responsible for the activation of ROS-generating enzymes and the formation of free radicals. Generally, there are two major mechanisms for mechanically induced intracellular Ca^{2+} mobilization: 1) Ca^{2+} influx through Ca^{2+}-permeable, stretch-activated (SA) channels and 2) Ca^{2+} release from intracellular Ca^{2+} stores (38, 51). Previously, we reported that SA channels play a crucial role in the mechanotransduction to cyclic stretch (1, 6, 18, 50). Cyclic stretch-induced Ca^{2+} influx through SA channels mediates O_{2}^{•-} production (1) and NF-κB activation (18). On the other hand, Pahl et al. (34) showed that both this signal cascade.

Reagents and chemicals. Gadolinium (III) chloride hexahydrate was purchased from Aldrich Chemical (Milwaukee, WI). Upon arrival, it was dissolved in distilled water at 1 M and stored at −80°C. Because Gd^{3+} is unstable, the concentrated Gd^{3+} was first diluted at 10 mM in distilled water and then diluted at the desired concentration in standard external solution (SES; in mM: 140 NaCl, 5 KCl, 2 CaCl_{2}, 10 glucose, and 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.40) just before use. Human plasma fibronectin was purified according to the method of Regnault et al. (36). PI3-kinase inhibitor (LY-294002) was purchased from Cell Signaling Technology (Beverly, MA). PLC inhibitor (U-73122) and the peptide Gly-Arg-Gly-Asp-Asn-Pro (GRGDNP) were acquired from Biomol International (Plymouth Meeting, PA). PKC inhibitor (H7), PKA inhibitor (H89), thapsigargin (TG), phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_{2}], and phosphatidylethanolamine (PE) were obtained from Sigma (St. Louis, MO). γ-[32P]ATP (3,000 Ci/mmol) and [γ-32P]PI(4,5)P_{2} (5.45 Ci/mmol) were purchased from Amersham Biosciences (Arlington Heights, IL). Mouse anti-focal adhesion kinase (FAK) monoclonal antibody, mouse anti-paxillin monoclonal antibody, and mouse anti-phosphotyrosine monoclonal antibody (clone PY20) were purchased from Transduction Laboratories (Lexington, KY). Glutathione-S-transferase (GST)-IκB-α (1–54) was a kind gift from Dr. Makoto Nakashima (Department of Biochemistry, Nagoya City University Medical School, Nagoya, Japan). Other chemicals used were of special grade.

Cell culture. Primary culture of human umbilical vein endothelial cells (HUVECs) was prepared from a human umbilical cord vein according to the method described by Shirinsky et al. (42). In brief, human umbilical cords were aseptically removed from the placenta just after birth. The veins were washed with phosphate-buffered saline (PBS; in mM: 137 NaCl, 8.10 Na_{2}HPO_{4}, 2.68 KCl, and 1.47 KH_{2}PO_{4}, pH 7.40), followed by treatment with 0.2% trypsin for 10 min. The perfusate was centrifuged at 200 g for 10 min. The resulting cells were washed with PBS again and then plated in 25-cm\(^2\) flasks and maintained in Humedia-EG2 medium (Kurabo, Osaka, Japan) supplemented with 10% fetal calf serum (FCS). HUVECs were incubated in a tissue culture incubator at 37°C in a humidified atmosphere of 5% CO_{2}-95% air. HUVECs used in this study were within two passages.

Stretch apparatus and cell treatments. The cells were stretched as previously described (21). Briefly, cells were removed from the flask with 0.01% EDTA-0.02% trypsin and transferred onto an elastic silicone (polydimethylsiloxane elastomer) chamber precoated with 50 μg/ml fibronectin (FN) for 12 h. After 24 h of incubation, HUVECs were found to be confluent. The medium was replaced with starvation medium [ Dulbecco’s modified Eagle’s medium (DMEM) containing 1% FCS, pH 7.4] 6 h before the cells were subjected to stretch. Both ends of the chamber were firmly attached to fixed metallic frames to produce a uniaxial continuous stretch by 150% of their original length. The stretched chamber with the frames was placed in an incubator. The silicone membrane was kept uniformly stretched over the whole membrane area during the incubation.

To examine the intracellular signal transduction pathway leading from the continuous mechanical stimulus, we switched starvation medium to DMEM containing 20 μM Gd^{3+}, a potent blocker for Ca^{2+}-permeable SA channels, or nominally Ca^{2+}-free medium (CFM; DMEM containing 5 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA)) just before the stretch. Inhibitors of several signaling pathways were preincubated in the following manner. Fifty millimolar LY-294002 (PI3-kinase inhibitor) was added to the serum-starved medium 1 h before the stretch. Fifty micromolar MG-132 (ER-resident Ca^{2+}-ATPase inhibitor), 5 μM U-73122 (PLC-γ inhibitor), 50 μM H-7 (PKC inhibitor), and 300 mM H89 [protein kinase A (PKA) inhibitor] were added 30 min before the stretch. Fifty micromolar GRGDNP, which competitively inhibits fibronectin binding, was added 3 h before the stretch application.

Determination of IKK activity. HUVECs were subjected to 150% continuous stretch for the indicated times after preincubation in starvation medium (DMEM with 1.0% FCS) for 6 h. For immunoprecipitation (IP), the cells were washed with ice-cold PBS and lysed in immunoprecipitated kinase buffer (50 mM HEPES, pH 8.0; 150 mM NaCl, 25 mM EGTA, 1 mM EDTA, 5 mM EGTA) just before the stretch. Inhibitors of several signaling pathways were preincubated in the following manner. Fifty millimolar PKC inhibitor, 50 mM H89, and 100 μM MTT were added 30 min before the stretch. Fifty micromolar GRGDNP, which competitively inhibits fibronectin binding, was added 3 h before the stretch application.

Measurement of IL-6 mRNA expression. HUVECs were subjected to 150% continuous stretch for the indicated times after preincubation in starvation medium for 6 h. Total RNA was extracted from stretched cells using the RNeasy Mini kit (Qiagen, Cologne, Germany). The samples were subjected to first-strand synthesis using oligo(dT)
primer and reverse transcriptase (Superscript II; Invitrogen, Carlsbad, CA). Real-time polymerase chain reaction (PCR) was performed using TaqMan Assays-on-Demand gene expression products and the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA) according to the manufacturer’s suggested protocol. Glycerinaldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control for each sample.

Western blot analysis. HUVECs were subjected to 150% continuous stretch for the indicated times after preincubation in starvation medium for 6 h. For immunoprecipitation, the cells were washed with ice-cold PBS and lysed using buffer A (10 mM Tris·HCl, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, and 1% SDS, pH 7.4). Cell lysates were centrifuged twice at 20,600 g for 20 min at 4°C. Two microfilters of 125-kDa monoclonal anti-FAK (40) or 68-kDa anti-paxillin antibody (47) were added, and the samples were incubated for 4°C for 1 h. Next, 20 μl of protein G-Sepharose were added, and the samples were incubated at 4°C for 1 h with gentle agitation. The resulting immunoprecipitates were washed five times with buffer A, and 30 μl of SDS sample buffer were added and boiled for 5 min. Proteins were then separated by SDS-PAGE and transferred electrophoretically onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA). The membranes were blocked with 5% BSA in PBS-Tween-20 (PBS containing 0.5% Tween 20) and subsequently probed with monoclonal antibodies in blocking buffer for 1 h at room temperature. The antibody-antigen complexes were detected using horseradish peroxidase-conjugated goat anti-mouse IgG (1:1,000 dilution). Immunoreactivity was determined using the ECL Plus enhanced chemiluminescence reaction system (Amersham, Little Chalfont, UK).

PKC kinase activity assay. HUVECs were subjected to 150% continuous stretch for the indicated times after preincubation in starvation medium for 6 h. A nonradioactive PKC assay was performed using a commercially available MESACUP kit (MBL, Nagoya, Japan). PKC present in samples catalyzed phosphorylation of phosphatidylserine (PS) peptide coated on microwells. The biotinylated monoclonal antibody 2B9 was bound to phospho-PS peptide and was subsequently detected with streptavidin conjugated to peroxidase. Peroxidase substrate was then added to the microwell, and the fluorescence intensity was measured photometrically at 492 nm.

Measurement of PIP2-specific PLC activity. HUVECs were subjected to a 150% continuous stretch for the indicated times after preincubation in starvation medium for 6 h. The cells were washed with ice-cold PBS twice and lysed using buffer B (in mM: 50 Tris·HCl, pH 7.2, 1 NaHCO3, 1 NaHSO3, 1 benzamidine, and 0.1 PMSF). The assay mixture for PIP2 hydrolysis (50 μl) contained an enzyme source (4 μM protein concentration measured using DC Protein Assay, Bio-Rad, Richmond, CA), 35 mM HEPES (pH 7.2), 0.04% octylglucoside, 0.4 mM DTT, 110 mM KCl, 3.2 mM EGTA, and 8.3 mM CaCl2 to obtain a 100 nM free Ca2+ concentration and sonicated micelles of 3 mM Pl(4,5)P2, 30 mM PE, and 3 mM [3H]P1(4,5)P2. An assay was performed at 30°C for 60 min and stopped by adding 0.25 ml of CHCl3/CH3OH/HCl (100:100:0.6 volume ratio) and 0.1 ml of 1 N HCl containing 5 mM EGTA. The mixture was centrifuged at 100 g for 5 min, and the radioactivity of the hydrophilic products and [3H]IP3 in the aqueous phase were determined. [Ca2+]i was measured using the fura-2 method with a fluorescence microscope system (ARUGAS/HiSCA; Hamamatsu Photonic KK, Hamamatsu, Japan) with a ×20 lens objective (Fluor 20; Zeiss) as described previously (32). Fluorescence ratio (R) was calculated using the following equation: \( R = \left( F_{340} - B_{340} \right) / \left( F_{380} - B_{380} \right) \), where \( F_{340} \) and \( F_{380} \) are the emission intensities at 510 nm excited at 340 and 380 nm, respectively, and \( B_{340} \) and \( B_{380} \) are the corresponding autofluorescence values. All experiments were performed at room temperature (22 ± 3°C).

Measurement of IL-6 concentrations. After preincubation with various inhibitors, the cells were subjected to 150% continuous stretch for 6 h and then the supernatants were collected and frozen. IL-6 concentration was measured using a two-step sandwich enzyme immunoassay (R & D Systems, Minneapolis, MN).

Statistical analysis. Results are expressed as means (SD). Statistical analysis was performed with paired and unpaired Student’s t-tests where appropriate. When analyzing two of three groups, P values were calculated according to Scheffe’s method for multiple comparisons. \( P < 0.05 \) was considered statistically significant.

RESULTS

Morphological changes in HUVECs. HUVECs cultured on an elastic silicone membrane coated with FN showed a cobblestone appearance in the nonstretched condition. When the membrane was stretched 150%, the cells on the membrane were stretched to the same extent (Fig. 1A). To estimate the magnitude of cell elongation, a length of the long axis of the cells was measured at various time points. The length was increased to 150% immediately after chamber stretch and maintained for 4 h after stretch onset (Fig. 1B). HUVECs preincubated with 50 μM GRGDNP, an α5β1 integrin-inhibitor peptide, for 3 h before stretch were also maintained at their stretched length for 4 h without significant cell detachment (data not shown).

IKK phosphorylation and IL-6 mRNA expression in response to continuous stretch. Analysis of the promoter region of the IL-6 gene revealed the presence of a binding site for NF-κB (24, 53). NF-κB is sequestered in a latent form in the cytoplasm by the interaction with the inhibitory IκB proteins. In response to proper signals, IκB is phosphorylated by activated IKK and degraded. This leads to the release of active NF-κB that eventually is translocated in the nucleus and binds to DNA (19, 41). We have previously reported that IL-6 secretion in response to continuous stretch is dependent on IL-6 gene transcription via sequential activation of IKK and NF-κB (22). The time course of IKK activation using an in vitro kinase assay demonstrated that IKK phosphorylation started at 5 min after continuous stretch and peaked at 15 min, followed by a gradual decrease (Fig. 2A). Furthermore, we examined the time course of IL-6 mRNA expression using quantitative real-time PCR. The result indicated that the transcription of the IL-6 gene peaked at 2 h after stretching and gradually attenuated (Fig. 2B).

Changes in [Ca2+]i. To examine whether uniaxial continuous stretch results in an increase in [Ca2+], in HUVECs, the cells cultured on an elastic silicone membrane were stretched by 150% continuously, and changes in [Ca2+]i were measured. As shown in Fig. 3A, in the presence of 2 mM extracellular Ca2+ (SES solution), onset of stretch elicited a transient increase in [Ca2+]i, that slowly declined to the initial [Ca2+]i level at ~3 min. To determine whether the increase in [Ca2+]i observed in response to sustained stretch originated from extracellular Ca2+, we measured [Ca2+]i, in cells stretched in SES containing 5 mM EGTA (CFM) or 20 μM Gd3+. A tiny
rise in [Ca\(^{2+}\)]\(_i\) that might be released from intracellular Ca\(^{2+}\) stores was observed when extracellular Ca\(^{2+}\) was depleted by EGTA (Fig. 3B) and when Ca\(^{2+}\) influx was blocked by Gd\(^{3+}\) (data not shown). To investigate a possible contribution of intracellular Ca\(^{2+}\) releases, intracellular Ca\(^{2+}\) stores were depleted by pretreating the cells with 5 μM TG, an ER Ca\(^{2+}\) pump inhibitor, for 30 min before the stretch. This treatment showed little effect on the stretch induced Ca\(^{2+}\) transient (Fig. 3C), however, suggesting that the stretch-induced Ca\(^{2+}\) transient was caused mainly by Ca\(^{2+}\) influx through SA channels. When extracellular Ca\(^{2+}\) was removed by EGTA after depletion of the intracellular Ca\(^{2+}\) stores by TG, the [Ca\(^{2+}\)]\(_i\) rise was almost completely inhibited (Fig. 3D).

Effects of [Ca\(^{2+}\)], on continuous stretch-induced IKK activation and IL-6 mRNA expression. To investigate whether the stretch-induced change in [Ca\(^{2+}\)] plays an important role for the activation of IKK and expression of IL-6 mRNA, we examined the effects of SA channel blocker, extracellular Ca\(^{2+}\) depletion, intracellular Ca\(^{2+}\) depletion, and extra- and intracellular Ca\(^{2+}\) depletion. Under these conditions, continuous stretch-induced IKK activation at 15 min, at which point IKK was maximally activated, was inhibited only when 5 μM TG
and 5 mM EGTA were coapplied (Fig. 4A). Similar to the results of IKK activation, IL-6 mRNA expression at 2 h, a peak time point of IL-6 mRNA expression, was inhibited only by the coapplication of TG and EGTA (Fig. 4B).

**Role of integrin in continuous stretch-induced IKK activation and IL-6 mRNA expression.** Integrins containing the β₁-subunit (e.g., α₅β₁) may function as mechanosensors by interacting with cognate ECM proteins (e.g., fibronectin) (43). We therefore examined the involvement of integrin (β₁-subunit) in stretch-induced IKK activation and IL-6 mRNA expression. First, to clarify whether integrin could be activated by uniaxial

![Figure 2](http://ajpcell.physiology.org/)  
**Fig. 2.** Activation of IkB kinase (IKK) phosphorylation and interleukin-6 (IL-6) mRNA expression in response to 150% uniaxial continuous stretch. A: autoradiogram of in vitro kinase assay of IKK-α using glutathione-S-transferase (GST)-IkB-α(1–54) and γ-[³²P]ATP as substrates at various time points. Bottom: activity of IKK relative to the nonstretched control. Each point represents the mean (SD) of four experiments. *P < 0.05 vs. nonstretched control.  
**B:** Changes in the amount of IL-6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA during continuous stretch were analyzed using quantitative real-time PCR. Relative expression levels of IL-6 mRNA normalized to GAPDH mRNA expression are shown. Each point represents the mean (SD) of four experiments. *P < 0.05 vs. nonstretched control.

![Figure 3](http://ajpcell.physiology.org/)  
**Fig. 3.** Changes in continuous-stretch induced increases in intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) under various pharmacological conditions. Confluent HUVECs in an elastic silicone chambers were subjected to 150% continuous stretch, and the change in [Ca²⁺]ᵢ level was measured as a ratio of 340- to 380-nm values (see EXPERIMENTAL PROCEDURES) in the presence of 2 mM extracellular Ca²⁺ (A), 5 mM EGTA (B), 5 μM thapsigargin (TG) (C), and a mixture of 5 μM TG and 5 mM EGTA (D). Each trace is representative of at least four independent repeatable experiments. Bottom traces indicate time course of stretch stimulus.
continuous stretch, we examined tyrosine phosphorylation of focal adhesion proteins (FAK and paxillin) that have been used to indicate integrin activation triggers intracellular TG-sensitive Ca2+ release that might be mediated by PLC-γ-dependent IP3 production.

Involvement of PI3-kinase, PLC-γ, and PKC in continuous stretch-induced IKK activation and IL-6 mRNA expression. To further characterize the signaling mechanisms responsible for continuous stretch-induced IKK activation and IL-6 mRNA expression, we examined the effects of inhibitors on the PI3-kinase-PLC-γ-PKC signaling pathway that may play a major role in the endothelial response to mechanical stress. As expected, 50 μM LY-294002 (PI3-kinase inhibitor), 5 μM U-73122 (PLC-γ inhibitor), or 50 μM H7 (PKC inhibitor) significantly attenuated the activation of IKK (Fig. 6A) and expression of IL-6 mRNA (Fig. 6B), while 300 nM H89 (PKA inhibitor) showed no significant effect on IKK activation and IL-6 mRNA expression in response to uniaxial continuous stretch in HUVECs (Fig. 6, A and B). These drugs showed little effect on IKK activity and IL-6 mRNA expression in unstretched HUVECs (control).

Involvement of integrin, PI3-kinase, PLC-γ, and [Ca2+]i in continuous stretch-induced PKC activity. Many investigators have demonstrated that mechanical forces, including shear stress and cyclic stretch, result in PKC activation in ECs (46, 52). Our results described above strongly suggest that PKC is also activated by continuous stretch in HUVECs. We therefore performed a nonradioactive PKC assay with and without the use of several inhibitors of the integrin-dependent signaling pathway. PKC was transiently activated at 10 min after stretch onset (Fig. 7A). This activation was significantly inhibited by several drugs, including GRGDNP, LY-294002, U-73122, or a mixture of TG and EGTA (Fig. 7B).

Involvement of integrin and PI3-kinase in continuous stretch-induced PIP2-specific PLC activity. In cardiomyocytes, mechanical stretch leads to activation of PLC (39). Therefore, we tested the effect of continuous stretch on PLC activity with varying stretch durations ranging from 3 to 30 min. The level of [3H]IP3 products of PIP(4,5)P2 hydrolysis increased in a time-dependent manner within 5 min of stretch. The PIP2-specific PLC activity increased markedly at ~5 min, keeping almost at its level for 30 min (Fig. 8A). The PIP2-specific PLC activity at 5 min was inhibited by GRGDNP or the PI3-K inhibitor LY-294002 (Fig. 8B).

Effects of various inhibitors on continuous stretch-induced IL-6 secretion. We have previously reported that IL-6 secretion in response to continuous stretch is markedly increased for the first 6 h and then gradually decreases and stabilizes for the next 12 h (22). To examine whether the various inhibitors tested above actually have effects on IL-6 production, we measured IL-6 concentration in the supernatant at 6 h after the onset of continuous stretch significantly increased for the presence of Gd3+, EGTA, TG, or H89 but not in the presence of GRGDNP, LY-294002, U-73122, or a coapplication of TG and EGTA, suggesting that IL-6 secretion requires an activa-
tion of integrin, PI3-K, PLC-γ, or PKC and a [Ca^{2+}] increase. Throughout the course of the present experiments, >90% of the cells looked healthy and were adherent to the membrane even over 6 h. Moreover, treatments with these drugs did not significantly affect the basal level of IL-6 secretion, strongly indicating that pharmacological results in this study were not contaminated by nonspecific cell damage.

DISCUSSION

In this study, we demonstrated that IL-6 secretion from HUVECs in response to continuous mechanical stretch requires outside-in signaling via integrins followed by activation of the PI3-kinase-PLC-γ-PKC signaling cascade that leads to IKK/NF-κB activation.

There is increasing evidence indicating that integrins are important in mechanotransduction in ECs (43). Evidence for shear stress-induced activation of integrins has been provided by several studies in which shear-induced responses were blocked by Arg-Gly-Asp (RGD) peptide. Preincubation of HUVECs with RGD peptide abolished shear stress-induced Akt phosphorylation (9) and secretion of basic fibroblast growth factor (17). Inspired by these studies, we have
investigated the contribution of integrins to continuous stretch-induced IL-6 production using RGD peptides. Our results demonstrate that the integrin-blocking RGD peptide (GRGDNP) attenuated IKK phosphorylation, IL-6 mRNA expression, and IL-6 secretion induced by continuous stretch. Although uniaxial continuous stretch caused a [Ca\(^{2+}\)]\(_i\) transient even in the presence of GRGDNP, probably via activation of SA channels, stretch-induced IL-6 production was not inhibited by the blockade of SA channels and external Ca\(^{2+}\) depletion. In contrast, inhibition of stretch-induced integrin activation by GRGDNP prevented IL-6 production. These results imply that the activation of integrins but not SA channels plays a pivotal role in eliciting continuous stretch-induced IL-6 secretion.

To clarify the downstream signaling molecules after integrin activation in response to continuous stretch, we investigated the involvement of PI3-kinase, PLC-\(\gamma\), and PKC using their inhibitors. All of the inhibitors impaired IKK activation, IL-6 mRNA expression, and IL-6 secretion in stretched HUVECs, suggesting that PI3-kinase, PLC-\(\gamma\), and PKC are necessary upstream effectors in IKK activation leading to IL-6 production. In addition, we examined the effects of these inhibitors on PKC and PLC-\(\gamma\) activities to confirm our hypothetical signaling pathway. Continuous stretch-induced PKC and PLC-\(\gamma\) activations were inhibited by an application of GRGDNP, LY-294002, or U-73122 and by GRGDNP or LY-294002, demonstrating that exposure of HUVECs to continuous stretch...
such channels have been found to induce Ca\(^{2+}\) influx through SA channels or Ca\(^{2+}\) release from intracellular stores is important in continuous stretch. In contrast, when both intra- and extracellular Ca\(^{2+}\) were depleted before stretching the cells, a sustained stimulus no longer elicited a [Ca\(^{2+}\)] rise, PKC or IKK activation, IL-6 mRNA expression, and IL-6 secretion. One possible interpretation of these results is that a [Ca\(^{2+}\)] increase, regardless of its source, is necessary for stretch-induced IL-6 production. However, as shown in Fig. 5A, treatment of the cells with the combination of EGTA and TG might cause an unphysiologically low [Ca\(^{2+}\)]], leading to deterioration of intracellular Ca\(^{2+}\) homeostasis. Nevertheless, we tentatively hypothesize that a [Ca\(^{2+}\)] increase would be necessary for the stretch-induced IL-6 secretion because [Ca\(^{2+}\)] increase is generally required for ROS-generating enzymes (e.g., PKC) and the formation of free radicals (1, 3, 34). As for the mechanism of the stretch-induced [Ca\(^{2+}\)] increase, we prefer the idea that an integrin-dependent Ca\(^{2+}\) release from intracellular stores is more essential than Ca\(^{2+}\) influx through SA channels, because inhibition of integrin activation almost completely abolished stretch-induced IL-6 production, while blockade of Ca\(^{2+}\) influx had little effect on it. As mentioned in RESULTS, the inhibition of integrin activation by GRGDNP eliminated a stretch-induced [Ca\(^{2+}\)], transient sensitive to TG. Whereas TG blocked an integrin-dependent intracellular Ca\(^{2+}\) releases, IL-6 production was not inhibited by TG treatment. Under this condition, Ca\(^{2+}\) influx might play a critical role in ROS generation leading to NF-κB activation.

Our previous study demonstrated that upregulation of IL-6 mRNA requires >30 min of sustained stretch (22), although the activation of IKK occurred within 15 min and subsided within 30 min. This discrepancy in the time courses implies that NF-κB translocation into the nucleus and subsequent IL-6 gene transcription may involve some unknown mechanisms that remained to be resolved.

In summary, our present results suggest that continuous stretch-induced IL-6 secretion in HUVECs is dependent on both outside-in signaling via integrins, which activate the PI3-K-PLC-γ-[Ca\(^{2+}\)] increase-PKC-IKK-NF-κB signaling pathway, leading to IL-6 secretion.

### Table 1. Effects of various inhibitors on continuous stretch-induced IL-6 release

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IL-6 concentration, pg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N-ST</td>
</tr>
<tr>
<td>None</td>
<td>37.5±8.3</td>
</tr>
<tr>
<td>Gd(^{3+}), 20 μM</td>
<td>28.1±16.4</td>
</tr>
<tr>
<td>EGTA, 5 μM</td>
<td>38.5±4.4</td>
</tr>
<tr>
<td>TG, 5 μM</td>
<td>38.2±12.5</td>
</tr>
<tr>
<td>TG + EGTA</td>
<td>26.7±2.4</td>
</tr>
<tr>
<td>GRGDNP, 50 μM</td>
<td>35.7±4.9</td>
</tr>
<tr>
<td>LY-294002, 50 μM</td>
<td>33.3±22.5</td>
</tr>
<tr>
<td>HT7, 50 μM</td>
<td>27.6±5.6</td>
</tr>
<tr>
<td>U-73122, 5 μM</td>
<td>26.6±7.1</td>
</tr>
<tr>
<td>H89, 300 nM</td>
<td>40.5±5.1</td>
</tr>
</tbody>
</table>

After preincubation with indicated dose of various inhibitors, 150% continuous stretch for 6 h was initiated. Continuous stretch-induced IL-6 concentrations in the supernatant concentration was measured for nonstretched and stretched cells. Results are expressed as means ± SD of 6 experiments. *P < 0.05 vs. nonstretched group. TG, thapsigargin; N-ST, nonstretch; ST, stretch.
ACKNOWLEDGMENTS

We are grateful to Dr. Makoto Nakanishi (Department of Biochemistry, Nagoya City University Medical School, Nagoya, Japan) for providing GST-IeB-(α,β).

GRANTS

This work was supported by Grants for Scientific Research 13480216 (to M. Sokabe) and 13671297 and 15591398 (to M. Nagino), Scientific Research on Priority Areas Grant 15086270 (to M. Sokabe), Creative Scientific Research Grant 16GS0308 (to M. Sokabe) from the Ministry of Education, Culture, Sports Science and Technology, and a grant from the Japan Space Forum (to M. Sokabe).

REFERENCES


Downloaded from hajlcell.org by 10.220.33.4 on June 20, 2017


