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Shear stress-induced c-*fos* activation is mediated by Rho in a calcium-dependent manner

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Abstract

We aimed at elucidating the molecular basis of c-*fos* promoter activation in vascular endothelial cells (ECs) in response to shear stress, with emphases on Rho family GTPases (Rho, Cdc42, and Rac) and intracellular calcium. Dominant-negative and constitutively activated mutants of these GTPases were used to block the action of upstream signals and to activate the downstream pathways, respectively. The role of intracellular calcium was assessed with intracellular calcium chelators. Only Rho, but not Cdc42 or Rac, is involved in the shear stress induction of c-*fos*. This Rho-mediated shear-induction of c-*fos* is dependent on intracellular calcium, but not on the Rho effector p160ROCK or actin filaments. While the inhibition of p160ROCK and its ensuing disruption of actin filaments decreased the basal c-*fos* activity in static ECs (no flow), it did not affect the shear-inductive effect. The calcium chelator BAPTA-AM inhibits the shear-induction, as well as the static level, of c-*fos* activity.
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Vascular endothelial cells (ECs), which form the inner lining of blood vessels at the blood–vascular interface, are constantly exposed to blood flow-associated mechanical forces. Shear stress is the tangential component of hemodynamic forces caused by a frictional drag of blood flowing over the EC surface [1]. Previous studies showed that shear stress activates multiple signaling pathways to modulate the expression of several genes in ECs [1–3]. With advances in the DNA microarray technology, recent endeavors have revealed the expression profiles of a variety of genes in ECs in response to sustained shear stress, providing significant insights into the role of shear stress in regulating EC functions and homeostasis [4–6]. For example, several genes related to inflammation and EC proliferation are downregulated in response to an arterial level of laminar shear stress at 12 dyn/cm^2 for 24 h [4]. This finding suggests that long-term exposure to laminar flow may keep ECs in a relatively noninflammatory and nonproliferate state, which is consistent with the clinical observation that ECs at the straight part of arteries are mostly in a quiescent state and are relatively protected from the development of atherosclerotic plaques [4,6]. While our knowledge in the genomic programming of ECs in response to shear stress has been greatly advanced by high-throughput genomic analysis, the regulatory mechanism underlying such biomechanical modulation of gene expression remains to be a central question. For example, shear stress causes a rapid and transient increase in the expression of the c-*fos* proto-oncogene [7–9], but the molecular basis underlying the shear-induced c-*fos* expression is not yet completely understood.

The c-*fos* gene is a member of the class of immediate-early genes. Its product, the c-Fos protein, is a major component of the activator protein 1 (AP-1) complex [10,11], which controls the basal and inducible expression of several genes, including those encoding for basic

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fibroblast growth factor (bFGF) and intercellular adhesion molecule-1 (ICAM-1). Shear stress has been shown to increase the transcriptional activities of bFGF and ICAM-1 genes in ECs [12,13]. Therefore, the shear-induced c-*fos* expression in ECs in the early time point may play an important role in modulating the expression of final target genes in delayed responses [2,8].

The c-*fos* gene is rapidly and transiently induced upon exposure of quiescent cells to a variety of stimuli. The transcriptional induction of the c-*fos* gene is independent of de novo protein synthesis, indicating that components for signal transduction cascades (i.e., second messengers and other signaling molecules) that target the c-*fos* promoter already exist [10,11]. In this study, we used a c-*fos* promoter model to investigate the molecular mechanism by which shear stress induces c-*fos* transcription, with emphases on Rho family small GTPases and intracellular calcium.

Rho family small GTPases, including Rho, Cdc42, and Rac, belong to the Ras superfamily of proteins that cycle between an active GTP-bound form and an inactive GDP-bound form, thereby functioning as molecular switches to turn on/off the downstream signal transduction processes [14,15]. Rho family GTPases regulate many essential cellular processes, including cytoskeleton organization, gene expression, and cell proliferation [14,15]. It has been shown that Rho, Cdc42, and Rac regulate the transcriptional activation of the serum response element (SRE) in the c-*fos* promoter through the serum response factor (SRF) [16]. Intracellular calcium also plays a critical role in many cellular events, including the modulation of transcription of c-*fos* by acting on the SRE and cyclic AMP response element (CRE) in its promoter region in a dose-dependent and cell type-specific manner [11,17,18]. It has been shown that shear stress regulates the activation of Rho family GTPases and the mobilization of intracellular calcium in ECs [1,19]. These findings led us to hypothesize that Rho family GTPases and intracellular calcium are involved in the signaling pathways by which shear stress activates the transcriptional activity of the c-*fos* promoter. To test this hypothesis, we used dominant-negative mutants and constitutively activated forms of small GTPases to block the action of upstream signals and to activate the downstream pathways, respectively. The role of intracellular calcium was assessed by using intracellular calcium chelators.

Materials and methods

Cell culture. Cell culture reagents were purchased from Gibco-BRL (Grand Island, NY) unless otherwise mentioned. Bovine aortic endothelial cells (BAECs) were isolated from the bovine aorta with collagenase as described [19] and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.292 mg/ml L-glutamine. Cell culture was maintained in a humidified 5% CO_2–95% air incubator at 37 °C. All experiments were conducted with cultures prior to passage 15.

Shear stress experiments. A recirculating flow system was used to impose shear stress on cultured BAECs as described [20]. In brief, a 75×38 mm glass slide with ECs formed the floor of a rectangular flow channel (0.025 cm in height, 2.5 cm in width, and 5.0 cm in length) created by sandwiching a silicone gasket between the glass slide and a custom-made acrylic plate. The channel has inlet and outlet for perfusing the cultured cells. A laminar shear stress of 12 dyn/cm^2 was generated by the flow resulting from the height difference between two reservoirs. During experiments, the flow system was kept at 37 °C in a constant-temperature hood and the circulation medium (DMEM supplemented with 10% FBS) was ventilated with a humidified gas mixture of 5% CO_2 and 95% air. The shear stress of 12 dyn/cm^2 is within the physiological range found in human major arteries and has been shown to activate EC signal transduction and induce the expression of several genes in vitro [3]. All shear stress experiments included static controls, i.e., ECs cultured on glass slides not exposed to shear stress.

DNA plasmids, transient transfection, and promoter activity assays. We used a c-*fos* promoter construct (i.e., c-*fos*-Luc) in which a luciferase reporter gene is driven by the 750-bp of the c-*fos* promoter, including the CRE (−63 to −54) and SRE (−319 to −300) [21]. DNA plasmids encoding the dominant-negative mutants hemagglutinin (HA)-RhoA(N19), HA-Cdc42(N17), and HA-Rac1(N17), and the dominant-active mutants myc-RhoA(V14), myc-Cdc42(V12), and myc-Rac1(V12) were gifts from Dr. Michael Karin (University of California, San Diego) and Dr. Martin Schwartz (University of Virginia) [22–25]. pSRα3 constructs were used as empty vector controls. In all experiments, the pSV-β-gal plasmid, which contains a β-galactosidase gene driven by the SV40 promoter and enhancer, was included in the transient transfection assays to monitor the transfection efficiency as described [26].

The various plasmids were transfected into BAECs at 70% confluence using the lipofectamine method (Gibco-BRL). After incubation for 6 h with transfection reagents, the cells were washed with DMEM and then incubated in fresh medium (DMEM supplemented with 10% FBS) to reach confluence. Within 48 h after transfection, the BAEC monolayers were subjected to shear stress or kept as static controls.

Reagents used in the following assays were purchased from Sigma (St. Louis, MO) unless otherwise mentioned. For promoter activity assays, plasmids encoding c-*fos*-Luc, pSV-β-gal, and mutants of Rho family GTPases were cotransfected into BAECs. After the experiments, the cells were washed with ice-cold phosphate-buffered saline (PBS) for three times and then lysed with a lysis buffer containing 0.1 M potassium phosphate (pH 7.9), 0.5% Triton X-100, and 1 mM DTT to release the luciferase and β-gal. Luciferase assays were carried out by adding a buffer containing 2 mM ATP, 0.075 mM luciferin, 100 mM Tricine, 10 mM Mg_2SO_4, 2 mM EDTA, and 1 mM DTT (pH 7.8) to the lysate and then measuring the total light output in a luminometer (Laboratorium Prof. Dr. Berthold GmbH, Germany). For β-gal activity assays, a buffer containing 1 mg/ml *o*-nitrophenyl-D-galactopyranoside in 0.1 M dibasic sodium phosphate (pH 7.3), 1.2 mM $MgCl_2$, and 60 mM β-mercaptoethanol was added to the lysate. After incubating the mixture at 37 °C for 30 min, 0.5 ml of 1 M Na_2CO_3 was added to terminate the reaction. The absorbance of the solution at 410 nm was determined by using a spectrophotometer (Model DU62, Beckman). In each experiment, the luciferase reporter activities of the samples were first normalized to the expressed β-gal levels for transfection efficiency. The relative luciferase activities thus obtained were further normalized to those of the pSRα3-transfected, static controls (set to be 1). Each experiment had duplicate or triplicate samples. Each set of data was obtained from at least three independent experiments.

Inhibitors. Y27632, a specific inhibitor of the Rho-associated kinase p160ROCK that mediates the Rho-regulation of actin-based cyto-

skeletal structure [27–29], was provided by Welfide (Osaka, Japan). 5,5′-difluoro-BAPTA/AM, an intracellular calcium chelator, was purchased from Calbiochem (La Jolla, CA). BAECs were incubated with Y27632 (10 μM) for 3 h or with BAPTA/AM (30 μM) for 30 min. The treated cells were washed three times with PBS to remove the inhibitors and then subjected to shear stress or kept as static controls.

Fluorescent labeling of actin. BAECs were washed with PBS, fixed in 4% paraformaldehyde in PBS for 10 min, followed by permeabilization with 0.5% Triton X-100 in PBS for 10 min. The specimens were then incubated in PBS containing 5 U/ml fluorescein isothiocyanate (FITC)-conjugated phalloidin (Molecular Probes, Eugene, Oregon) for 1 h. Fluorescently labeled actin-based cytoskeletal structure was observed under an inverted Nikon microscope equipped with epifluorescence optics.

Data presentation. Bar graphs represent means ± standard error of the mean from at least three separate experiments. Statistical analysis was performed by using the commercial software StatView (Abacus Concepts, Berkeley, CA), in which the Student t test and analysis of variance (i.e., ANOVA) were used to determine the significance of differences between two sets of experiments and between more than two sets of experiments, respectively. A value of $P < 0.05$ was considered statistically significant.

Results

Shear stress increased the c-fos promoter activity

Within 48 h after the cotransfection of plasmids encoding c-*fos*-Luc and pSV-β-gal, the BAECs were either kept as static controls or exposed to shear stress at 12 dyn/cm^2 in the flow system, followed by luciferase and β-gal assays. The c-*fos* promoter activity in ECs was indicated by the luciferase activity normalized with the expressed β-gal level. Fig. 1A shows that the luciferase activity was significantly increased to 2.38 (±0.23)-fold and 2.42 (±0.18)-fold in ECs subjected to shear stress for 3 and 5 h, respectively, when compared to that of static controls (set to be 1). The increase in luciferase activity after 1- or 2-h shearing was not significant (data not shown). Therefore, a shearing time of 3 h was chosen for the following experiments. The shear-induced luciferase activity was always higher than 1.5-fold.

Effects of mutants of Rho family small GTPases on the shear-induced c-fos promoter activation

In the absence of extracellular stimuli, constitutively activated Rho, Cdc42, or Rac alone was shown to be sufficient to upregulate the transcriptional activity of SRE in the c-*fos* promoter through SRF in fibroblast-like cells under static conditions [16]. We have found that shear stress increases the activities of Rho [19], Cdc42 [19], and Rac (Y.T. Shiu, S. Li, S. Chien, unpublished data) in BAECs. To test whether Rho family GTPases are involved in the shear-activation of the c-*fos* promoter, BAECs were cotransfected with plasmids encoding c-*fos*-Luc, pSV-β-gal, and one of the mutant forms of these GTPases, i.e., the dominant-active mutants myc-RhoA(V14), myc-Cdc42(V12), and myc-Rac1(V12), and the dominant-negative mutants HA-RhoA(N19), HA-Cdc42(N17), and HA-Rac1(N17) [22–25]. Fig. 1B shows that, under static conditions, overexpression of any constitutively activated form of Rho family GTPases in ECs significantly increased the luciferase activity when compared to that of the control cells transfected with pSRα3 empty vector (set to be 1). Shear stress augmented luciferase activities in the pSRα3- and active GTPase-transfected ECs to similar levels ($p > 0.05$ among sheared samples by ANOVA).

As shown in Fig. 1C, overexpression of dominant-negative mutants RhoA(N19) or Rac1(N17) in ECs caused an approximately 50% decrease in the luciferase activity under static conditions when compared to that of the pSRα3-transfected controls, whereas Cdc42(N17) had little effect. These data suggest that functional Rho and Rac are required to maintain the basal activity of the c-*fos* promoter in static ECs. In response to fluid shearing, luciferase activities were elevated in the pSRα3-, Cdc42 (N17)-, or Rac1(N17)-transfected ECs to similar levels ($p > 0.05$ among these sheared samples by ANOVA). The shear induction was abolished only in the RhoA(N19)-transfected ECs. The results were similar whether serum was present or absent in the perfusion medium. Overall, these data indicate that functional Rho is necessary for the shear-induced c-*fos* promoter activation. We then focused on the effectors downstream to Rho.

Role of Rho-regulated cytoskeleton organization in the shear-induced c-fos promoter activation

Rho has many downstream effectors that are involved in various signaling pathways [14,15]. It has been shown that p160ROCK, a Rho-associated serine/threonine kinase belonging to the Rho-kinase family, is involved in the Rho regulation of actin-based cytoskeletal structure [27,28]. Since actin dynamics was found to mediate the activation of SRF by Rho [30,31], we investigated whether p160ROCK is a downstream effector of Rho in the shear-induced c-*fos* promoter activation. BAECs were cotransfected with plasmids encoding c-*fos*-Luc and pSV-β-gal as described above. The cells were treated with Y27632, a specific inhibitor of p160ROCK [29], at 10 μM for 3 h before exposure to flow. Fig. 2A shows that this treatment disrupted the EC stress fibers, which did not recover following 3-h shearing or static incubation in fresh medium. Although Y27632 caused an approximately 30% decrease in the luciferase activity in ECs under static conditions (Fig. 2B), shear stress caused a marked increase in the luciferase activity of these Y27632-treated ECs. In concert with the finding that the shear-induced c-*fos* promoter activation was not affected by the absence of stress fibers, we found that cytochalasin D (an actin filament disrupting drug) and the transfection of dominant-negative mutants of

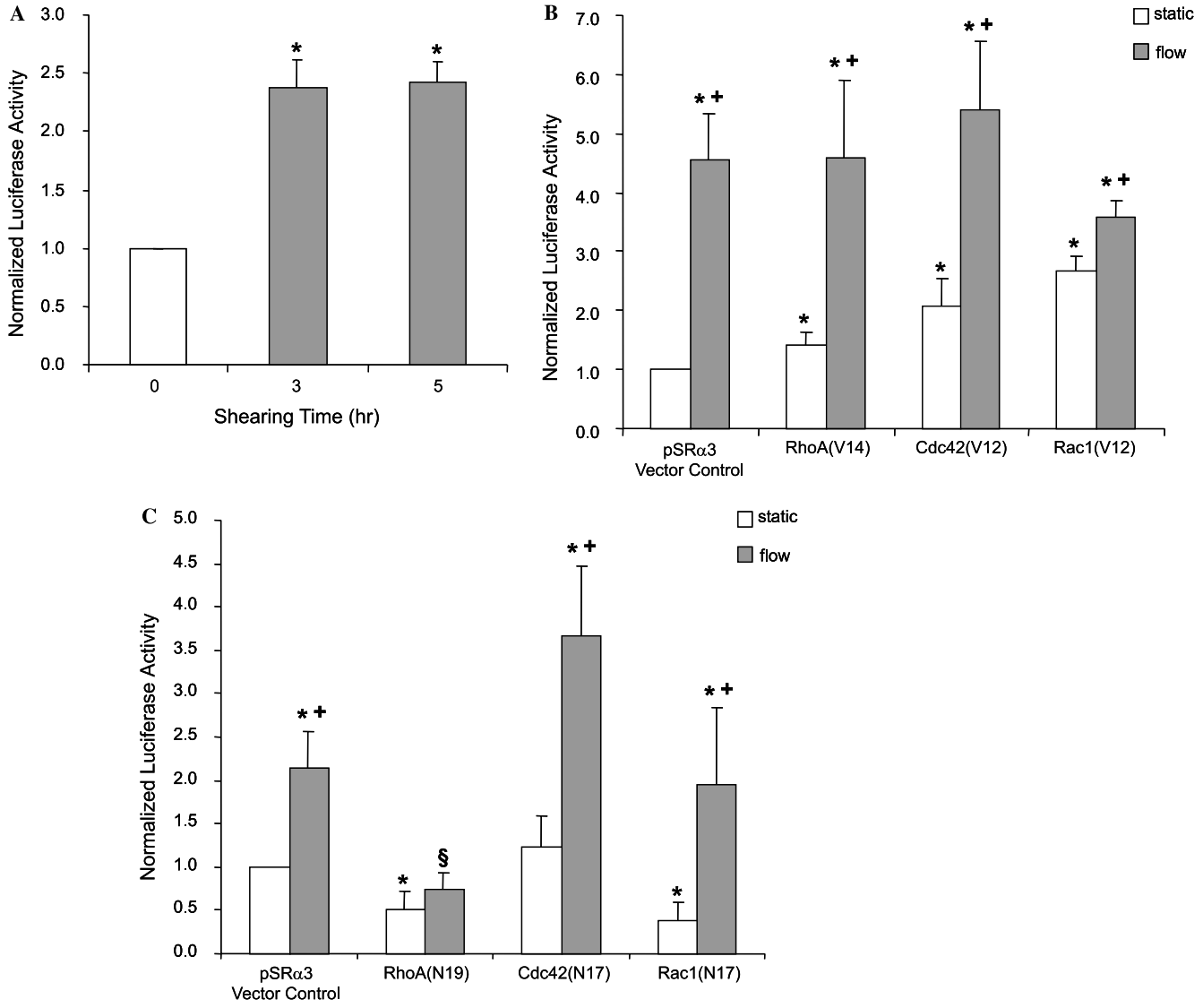

Fig. 1. Shear stress increased the c-*fos* promoter activity through Rho GTPase. (A) c-*fos* promoter activation in response to shear stress (12 dyn/cm^2) applied for 3 and 5 h. BAECs were cotransfected with plasmids encoding c-*fos*-Luc (1 μg/slide) and pSV-β-gal (0.5 μg/slide), and then kept as static controls or exposed to shear stress, followed by luciferase and β-gal assays. The results of luciferase assays were first normalized to the expressed β-gal levels for transfection efficiency and further normalized to those of static controls (set to be 1). *$p < 0.05$ when compared with static controls (0 h). (B) BAECs were cotransfected with plasmids encoding c-*fos*-Luc (1 μg/slide), pSV-β-gal (0.5 μg/slide), and one of the constitutively activated forms of Rho family GTPass (2.5 μg/slide), i.e., myc-RhoA(V14), myc-Cdc42(V12), or myc-Rac1(V12). pSRα3 constructs were used as vector controls. The transfected cells were kept as static controls or exposed to shear stress at 12 dyn/cm^2 for 3 h, followed by luciferase and β-gal assays. The results of luciferase assays were first normalized to the expressed β-gal levels and further normalized to those of the vector controls under static conditions (set to be 1). *$p < 0.05$ when compared with vector controls under static conditions. +$p < 0.05$ when compared with respective static controls with the same plasmids. (C) BAECs were cotransfected with plasmids encoding c-*fos*-Luc (1 μg/slide), pSV-β-gal (0.5 μg/slide), and one of the dominant-negative mutants of Rho family GTPass (2.5 μg/slide), i.e., HA-RhoA(N19), HA-Cdc42(N17), or HA-Rac1(N17). pSRα3 constructs were used as vector controls. The transfected cells were kept as static controls or exposed to shear stress at 12 dyn/cm^2 for 3 h, followed by luciferase and β-gal assays as described in (B). * and +, same as in (B). §$p < 0.05$ when compared with vector controls under flow conditions.

p160ROCK into BAECs also had little effect on this shear-induction, even though they decreased the basal level of the luciferase activity under static conditions (data not shown). Taken together, these results suggest that the action of Rho on p160ROCK and the cytoskeleton is not a primary factor in the shear-induced transcriptional activity of the c-*fos* promoter.

Role of intracellular calcium in the shear-induced c-fos promoter activation

Rho has been shown to regulate the mobilization of intracellular calcium through PIP2 kinase or phospholipase Cγ [32,33]. The transcriptional activity of the c-*fos* gene is regulated by calcium in a dose-, source-, and cell

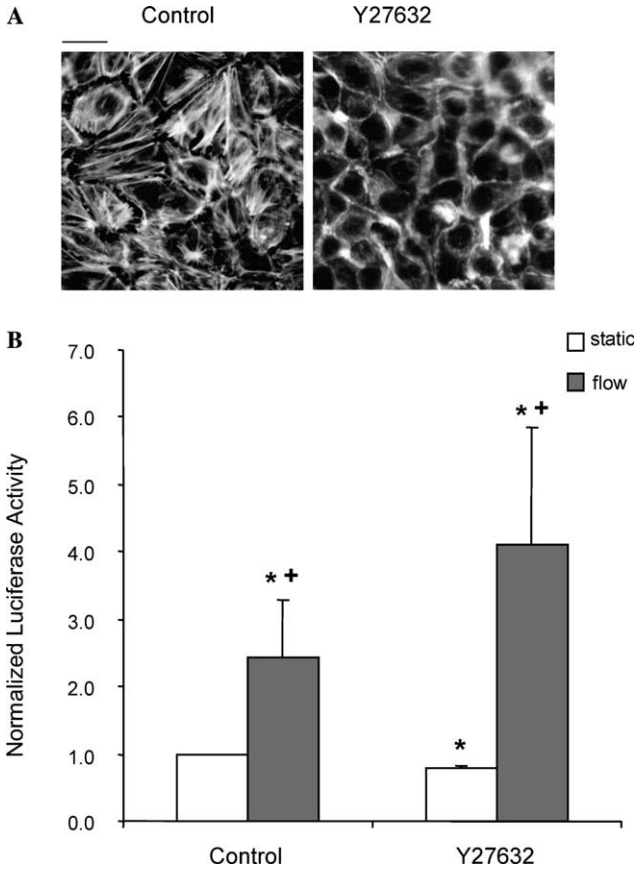

Fig. 2. Rho-associated kinase p160ROCK was not involved in the Rho-mediated c-*fos* activation by shear stress. (A) BAECs were cotransfected with plasmids encoding c-*fos*-Luc (1 μg/slide) and pSV-β-gal (0.5 μg/slide). The cells were treated with Y27632, a specific inhibitor of p160ROCK, at 10 μM for 3 h, followed by fluorescent labeling of the actin-based cytoskeletal structure by FITC-conjugated phalloidin. Stress fibers in ECs were disrupted by Y27632. Bar represents 30 μm. (B) BAECs were cotransfected with plasmids encoding c-*fos*-Luc (1 μg/slide) and pSV-β-gal (0.5 μg/slide), and were treated with Y27632 at 10 μM for 3 h. The treated cells were kept as static controls or exposed to shear stress at 12 dyn/cm^2 for 3 h, followed by luciferase and β-gal assays. The results of luciferase assays were first normalized to the expressed β-gal levels and further normalized to those of static controls without Y27632 treatment (set to be 1). *$p < 0.05$ when compared with static controls without Y27632 treatment. +$p < 0.05$ when compared with static controls with Y27632 treatment.

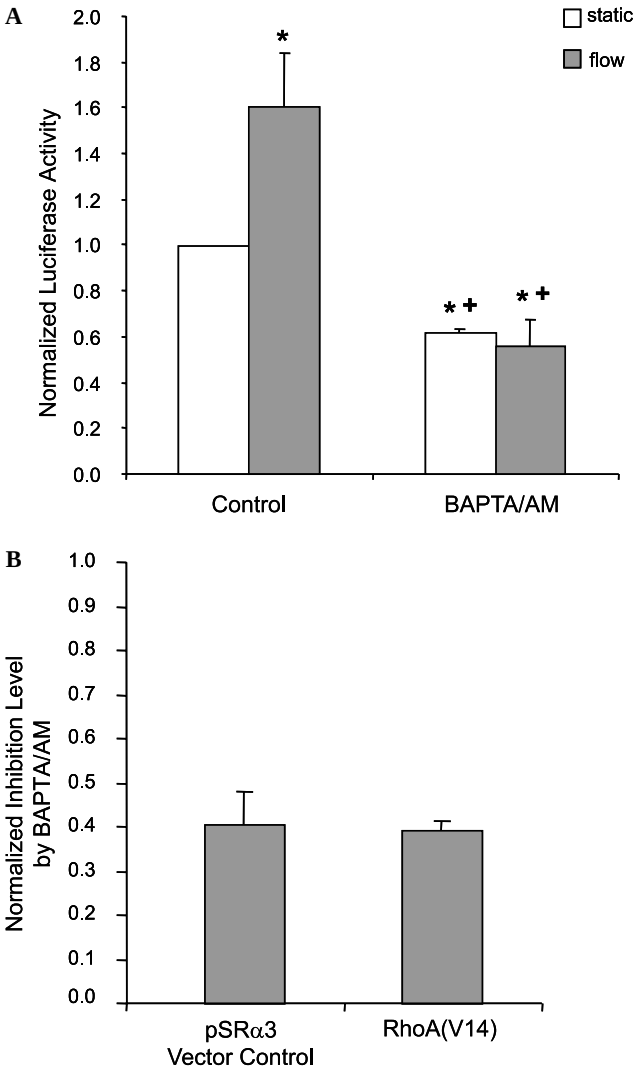

Fig. 3. Intracellular calcium was involved in the Rho-mediated c-*fos* activation by shear stress. (A) BAECs were cotransfected with plasmids encoding c-*fos*-Luc (1 μg/slide) and pSV-β-gal (0.5 μg/slide), and were treated with BAPTA/AM, an intracellular calcium chelator, at 30 μM for 30 min. The treated cells were kept as static controls or exposed to shear stress at 12 dyn/cm^2 for 3 h, followed by luciferase and β-gal assays. The results of luciferase assays were first normalized to the expressed β-gal levels and further normalized to those of static controls without BAPTA/AM treatment (set to be 1). *$p < 0.05$ when compared with static controls without BAPTA/AM treatment. +$p < 0.05$ when compared with sheared samples without BAPTA/AM treatment. (B) BAECs were cotransfected with plasmids encoding c-*fos*-Luc (1 μg/slide), pSV-β-gal (0.5 μg/slide), and pSRα3 as vector controls or the constitutively activated form of Rho (2.5 μg/slide), i.e., myc-RohA(V14). The transfected cells were treated with BAPTA/AM at 30 μM for 30 min, followed by luciferase and β-gal assays. The results of luciferase assays were first normalized to the expressed β-gal levels. The inhibition level was determined as the ratio of the luciferase activities in ECs transfected with the same plasmids with vs. without BAPTA/AM treatment.

type-dependent manner [11,17,18]. We tested whether intracellular calcium is involved in the Rho-mediated c-*fos* activation by shear stress. BAECs were cotransfected with plasmids encoding c-*fos*-Luc and pSV-β-gal as described above, and then treated with BAPTA/AM, an intracellular calcium chelator that does not affect the activation of Rho [34], at 30 μM for 30 min before the experiments. As shown in Fig. 3A, BAPTA/AM caused a 40% decrease in luciferase activity in static controls, and it completely abolished the shear-induction of the c-*fos* promoter. This is consistent with a previous finding that intracellular calcium is required in the shear-induced expression of the endogenous c-*fos* gene in human umbilical vein ECs [8]. The treatment of BAECs with DMSO, a solvent control of BAPTA/AM, did not affect

the luciferase activity as compared to BAECs untreated with DMSO.

The inhibitory effect of BAPTA/AM was next tested in BAECs cotransfected with plasmids encoding c-*fos*-Luc, pSV-β-gal, and either the constitutively activated form of Rho, i.e., myc-RhoA(V14), or pSRα3 as vector controls. As mentioned above, the luciferase activity in ECs under static conditions was increased by overexpression of myc-RhoA(V14). To normalize for the difference in the basal activity, the inhibition level was determined as the ratio of the luciferase activities in ECs transfected with the same plasmids with and without BAPTA/AM treatment. We found that BAPTA/AM significantly attenuated the luciferase activity in the pSRα3- and myc-RhoA(V14)-transfected ECs to 0.40 (±0.08)-fold and 0.39 (±0.03)-fold, respectively (Fig. 3B). These data suggest that the Rho-mediated c-*fos* promoter activation is a calcium-dependent process under both basal and RhoA(V14)-stimulated states.

Discussion

The major finding of this study is the identification of Rho GTPase as a signaling molecule in the shear-modulated EC gene expression. Furthermore, the Rho-mediated c-*fos* promoter activation by shear stress is dependent on intracellular calcium, but not on actin-based cytoskeleton.

The temporal pattern of endothelial gene expression in response to shear stress shows that the transcriptional activities of several immediate-early genes are rapidly induced in ECs upon exposure to fluid shearing [1,2]. Among these mechano-sensitive immediate-early genes, the c-*fos* and c-*jun* proto-oncogenes encode DNA binding proteins c-Fos and c-Jun, respectively. The AP-1 complex is composed of either c-Jun/c-Fos or c-Jun/c-Jun dimers, with the former having a higher DNA binding affinity and hence a greater biological activity than the latter [10]. Static ECs (no exposure to flow) were shown to have low c-Fos protein levels but very high c-Jun protein levels [9], possibly because c-*jun* transcripts are more stable than the rapidly-degraded c-*fos* mRNA [10]. This may explain the greater shear-inductive effect on the transcription of c-*fos* than c-*jun* [8,9]. The dramatic increase in c-*fos* expression in ECs exposed to shear stress suggests a change in the DNA binding affinity, as well as the total amount, of AP-1 [8]. A consensus DNA binding site for AP-1 has been identified in the positive and negative regulatory regions of several genes. Therefore, the transcribed products of c-*jun* and c-*fos,* especially the latter, may act as mechano-sensitive transcription factors to modulate EC gene expression in response to shear stress [2,8].

The induced expression of the immediate-early gene c-*fos* is usually rapid (within minutes) and transient in various cell types following exposure to phorbol ester 12-*O*-tetradecanoylphorbol-13-ester (TPA), growth factors, neurotransmitters, shear stress, etc. [8,10,11]. The SRE and CRE in the c-*fos* promoter have been identified as major targets for stimulatory signals. The c-*fos* SRE forms a ternary complex with the transcription factors SRF and ternary complex factor (TCF). SRF is ubiquitously expressed and binds as a dimer to the c-*fos* SRE. By itself, SRF can mediate transcriptional activation, whereas TCF interacts with the c-*fos* SRE only if the SRE is already occupied by SRF. The SRE is necessary and sufficient for rapid induction of the c-*fos* gene by serum, growth factors, and phorbol ester TPA [11]. It has been reported that cyclic AMP inhibitors do not inhibit the expression of the endogenous c-*fos* gene induced by shear stress [8], suggesting that SRE, but not CRE, is involved in the shear induced c-*fos* activation. Therefore, the following discussion is focused on the SRE.

Rho family GTPases, including Rho, Cdc42, and Rac, have been reported to regulate the transcriptional activation of SRE through SRF in a manner that is independent of pathways mediated by mitogen-activated protein kinases [16]. In the absence of extracellular signals, overexpression of constitutively activated Rho, Cdc42, or Rac alone is sufficient to induce SRE activation in fibroblast-like cells under static conditions [16]. Although they all act through SRF, it was found that the Rho-SRF pathway and the Cdc42/Rac-SRF pathway are independent of each other in converging on the SRE [16]. The relation between these two parallel pathways and the detailed signaling transduction processes between Rho family GTPases and SRF are not yet clearly understood. We found that overexpression of the constitutively activated form of Rho or Rac increased the c-*fos* promoter activity in BAECs under static conditions, whereas their dominant-negative mutants decreased c-*fos* activity, suggesting that functional Rho and Rac are required to maintain the basal c-*fos* activity in static ECs. Cdc42 was not found to be necessary for the basal c-*fos* activity in our system, though it was in other studies using fibroblast-like cells [35], possibly due to the differences in cell types.

It has been shown that shear stress activates Rho family GTPases in ECs [19], suggesting that these GTPases may be the mediators in the shear induction of c-*fos*. The results of this study show that only Rho, but not Cdc42 or Rac, is involved in the shear stress induction of c-*fos*. To study the downstream effectors of Rho, we first investigated the engagement of cytoskeleton because it is known that cytoskeletal rearrangements are involved in c-*fos* expression [30,31] and that Rho plays an important role in the regulation of actin-based cytoskeletal structure through downstream effectors

such as p160ROCK [27,28]. The basal c-*fos* level in ECs under static conditions was decreased by a specific inhibitor of p160ROCK (i.e., Y27632) [29], but the shear-inductive effect on c-*fos* was not affected by Y27632. While the disruption of actin filaments by Y27362 and the actin depolymerizing agents cytochalasin D (capping of actin filament barbed-ends) and latrunculin B (sequestration of actin monomers) has been shown to abolish the induction of c-*fos* transcription by chemical stimuli such as angiotensin II [36,37], insulin [38], and IL-1β [39], the present findings show that the absence of actin filaments did not abolish the induction of c-*fos* by shear stress. These results indicate the uniqueness of the shear-regulation of c-*fos* transcription. Although we cannot rule out the involvement of cytoskeleton completely, our results indicate the involvement of other more dominant signaling pathway(s).

Rho has been shown to regulate the mobilization of intracellular calcium, which is a potent second messenger in modulating the transcriptional activity of the c-*fos* gene [11,18,32,33]. The chelation of intracellular calcium by BAPTA/AM does not affect the activation of Rho [34]. Hence we used BAPTA/AM to access the role of intracellular calcium in the Rho-mediated shear induction of *c-fos*. We found that BAPTA/AM not only decreased the basal level of the c-*fos* activity under static conditions, but also abolished the shear-inductive effect on the c-*fos* promoter. Furthermore, BAPTA/AM caused a significant decrease (by approximate 60%) in c-*fos* activity in ECs cotransfected with constitutively activated Rho or empty vectors, suggesting that intracellular calcium plays a significant role in the Rho regulation of c-*fos* activation. Thus, we conclude that the signal transduction process for the shear stress induction of c-*fos* activation is mediated by Rho GTPase in a calcium-dependent manner.

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