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The Ras-JNK Pathway Is Involved in Shear-Induced Gene Expression

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Hemodynamic forces play a key role in inducing atherosclerosis-implicated gene expression in vascular endothelial cells. To elucidate the signal transduction pathway leading to such gene expression, we studied the effects of fluid shearing on the activities of upstream signaling molecules. Fluid shearing (shear stress, 12 dynes/cm^2 [1 dyne = 10^{-5} N]) induced a transient and rapid activation of p21^{ras} and preferentially activated c-Jun NH_2 terminal kinases (JNK1 and JNK2) over extracellular signal-regulated kinases (ERK-1 and ERK-2). Cotransfection of RasN17, a dominant negative mutant of Ha-Ras, attenuated the shear-activated JNK and luciferase reporters driven by 12-*O*-tetradecanoylphorbol-13-acetate-responsive elements. JNK(K-R) and MEKK(K-M), the respective catalytically inactive mutants of JNK1 and MEKK, also partially inhibited the shear-induced luciferase reporters. In contrast, Raf301, ERK(K71R), and ERK(K52R), the dominant negative mutants of Raf-1, ERK-1, and ERK-2, respectively, had little effect on the activities of these reporters. The activation of JNK was also correlated with increased c-Jun transcriptional activity, which was attenuated by a negative mutant of Son of sevenless. Thus, mechanical stimulation exerted by fluid shearing activates primarily the Ras-MEKK-JNK pathway in inducing endothelial gene expression.

Vascular endothelial cells (EC), located at the interface between the blood and the vessel wall, are exposed to the mechanical environment resulting from hemodynamic activities. Fluid shear stress is the hemodynamic force acting tangentially on the vascular EC, and it plays significant roles in atherogenesis and reperfusion injury. Many genes encoding growth factors (e.g., platelet-derived growth factor and transforming growth factor β-1), vasoconstrictors (e.g., endothelin 1), vasodilators (e.g., nitric oxide synthase), adhesion molecules (e.g., intercellular adhesion molecule 1), and monocyte chemoattractants (e.g., monocyte chemotactic protein 1 [MCP-1]) in EC are modulated by fluid shearing (see reference 7 for a review). The induction of some, and perhaps the majority, of these inflammation-related genes is rapid and transient, and de novo protein synthesis is not required. These are the characteristics of the expression of immediate-early (IE) genes induced by mitotic factors and agonists. The 12-*O*-tetradecanoylphorbol-13-acetate-responsive element (TRE) mediates the expression of many IE genes through its interaction with the transcription factor AP-1, a Jun-Fos heterodimer, or a Jun-Jun homodimer (1). We have found that a divergent TRE in the 5′ promoter region of the MCP-1 gene is responsible for its mechanical inducibility (41). A consensus TRE with the sequence TGACTACA is sufficient for shear-induced reporter activities in different types of cells. The applied fluid shearing probably exerts its actions on the cellular membrane to initiate biochemical signals which can then be transduced through the cytoplasm into the nucleus, where the activation of AP-1–TRE occurs.

A major process through which extracellular stimuli can be transmitted into cells involves membrane-associated p21^{ras} and its downstream cytoplasmic kinase pathways, especially the members of the mitogen-activated protein kinase (MAPK) family. p21^{ras} is a small GTPase molecule that plays a key role in the signal transduction pathways of cellular responses to stimuli by mitogens, cytokines, environmental stresses, and UV irradiation (see reference 20 for a review). p21^{ras} cycles between an active GTP-bound state and an inactive GDP-bound state, thereby functioning as a molecular switch in response to extracellular stimuli for the control of normal and transformed cell growth (see reference 3 for a review). Activated p21^{ras} triggers two protein kinases, Raf-1 and MEK (MAPK kinase) kinase (MEKK), which activate the downstream MAPKs, including c-Jun NH_2-terminal kinases (JNK) and extracellular signal-regulated kinases (ERK) (11, 33). Raf-1 activates ERK but not JNK, whereas MEKK mediates preferentially JNK over ERK (33, 48). In different types of cells in response to UV irradiation, Ha-Ras expression, and osmotic shock, JNK kinase activates JNK by phosphorylating the Thr-Pro-Tyr phosphorylation sites, and the activated JNK binds to c-Jun to specifically phosphorylate Ser-63 and Ser-73 at the N terminus (10, 29). In response to Ha-Ras expression, serum growth factor, or stimulation with the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate, MEK activates ERK, which in turn phosphorylates the transcription factor p62 ternary complex factor (p62^{TCF}), leading to the activation of c-Fos (5, 18, 30, 37). In REF-52 fibroblasts, the activation of AP-1–TRE by these stimuli is mediated through ERK (16).

While several laboratories, including ours, have shown that fluid shearing induces a variety of transient responses in the endothelial cytoplasm and nucleus, there is little, if any, knowledge on how EC transduce the mechanical stimuli into biochemical signals which ultimately activate downstream gene expression. We report here that fluid shearing, a physiological form of hemodynamic forces, activates p21^{ras} in EC in a rapid and transient manner and that this activation is followed by the activation of the MEKK-JNK pathway, leading to the induction of AP-1–TRE-mediated gene expression in the nucleus. In contrast, the ERK pathway is weakly activated by fluid shearing and is not essential for the shear-induced activation of AP-1–TRE. These results indicate that hemodynamic forces

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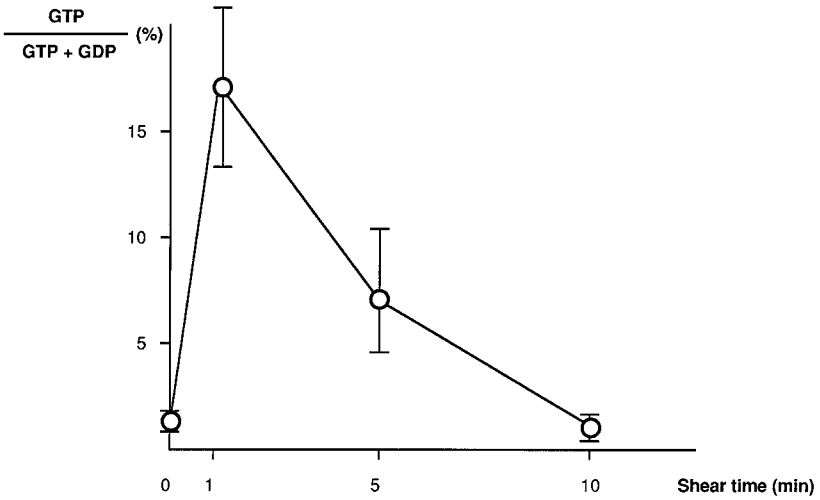

FIG. 1. Fluid shearing increases the ratio of p21^{ras} · GTP to p21^{ras} · GDP. Monolayers of ^{32}P-labeled BAEC were either kept as static controls (time zero) or subjected to a fluid shearing of 12 dynes/cm^2 for the periods of time indicated. Guanine nucleotides were then eluted from the cell lysate and separated by thin-layer chromatography. The ratio of GTP to GTP plus GDP was determined by densitometry. The results are the means ± standard deviations from three experiments.

have the same signaling pathways as a variety of stimuli, including osmotic pressure, chemical stress, and UV irradiation, for activating the promoter regions of IE genes.

MATERIALS AND METHODS

Cell cultures and fluid shearing experiments. Bovine aortic EC (BAEC) prior to passage 10 were used in all the experiments. The cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. All cell cultures were kept in a humidified 5% CO_2–95% air incubator at 37°C. BAEC were cultured on slides (38 by 76 mm) to confluence, and the slides were then assembled into a rectangular flow channel with a height of 270 μm, through which the medium flowed. The system was tightly sealed by using a silicon gasket and a vacuum line. A surface area of 14 cm^2 on the BAEC-seeded slide, confined by the gasket, was exposed to the applied fluid shearing, which was generated by circulating the tissue culture medium through a hydrostatic pump connected to upper and lower reservoirs (15). The pH of the system was kept constant by gassing with 95% air–5% CO_2, and the temperature was maintained at 37°C by immersing the flow system in a water bath. The shear stress, determined by the flow rate perfusing the channel and the channel thickness, was 12 dynes/cm^2 (1 dyne = 10^{-5} N), which is comparable to the physiological range in the human major arteries and which has been found to induce the expression of many IE genes in vitro (22, 40). The duration of the applied fluid shearing was 8 h in the gene regulation experiments and varied from 1 to 60 min in the signal transduction experiments. Static control experiments were performed on BAEC on slides not exposed to fluid shearing.

p21^{ras} guanidine nucleotide binding assay. The assays were performed according to the procedures described previously by Downward et al. (12), with minor modifications. BAEC cultured on a glass slide were labeled with 0.5 mCi of [^{32}P]P_i (ICN Radiochemicals) per ml for 6 h in a phosphate-free medium. After being labeled, the cells were subjected to fluid shearing or kept as static controls. Cell extracts were then prepared by lysing the BAEC in a buffer containing 50 mM Tris-HCl (pH 7.5), 0.5% Nonidet P-40, 0.15 M NaCl, 0.1 mM Na_3VO_4, 20 mM $MgCl_2$, 0.5% Triton X-100, 1 μg of leupeptin per ml, 1 mM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol (DTT), and 2 mM β-glycerolphosphate. Ras proteins were immunoprecipitated with rat anti-p21^{ras} monoclonal antibody (Santa Cruz). The bound guanine nucleotides were eluted from the precipitated protein complexes by using a buffer containing 20 mM Tris-HCl (pH 7.5), 20 mM EDTA, 2 mM DTT, 2% sodium dodecyl sulfate (SDS), and 2 mM GTP. The eluted nucleotides were separated by thin-layer chromatography using polyethyleneimine-cellulose plates with 0.75 M K_2HPO_4, pH 3.4. The GDP and GTP contents were assessed by autoradiography.

Assays of ERK and JNK activities. Five micrograms of anti-MAPK–protein A–agarose (Upstate Biotechnology), in a buffer containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.7), 75 mM NaCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 0.05% Triton X-100, 0.5 mM DTT, 20 mM β-glycerolphosphate, 0.1 mM Na_3VO_4, 2 μg of leupeptin per ml, and 100 μg of phenylmethlysulfonyl fluoride per ml, was added to the cell lysate to immunoprecipitate ERK. The suspension was mixed at 4°C for 4 h and centrifuged. The pelleted beads were washed in phosphate-buffered saline (PBS) containing 0.1% Triton X-100 and then resuspended in 30 μl of a kinase buffer which contained 20 mM HEPES (pH 7.6), 20 mM $MgCl_2$, 20 mM β-glycerolphosphate, 20 mM *p*-nitrophenyl phosphate, 0.1 mM Na_3VO_4, 2 mM DTT, 20 μM ATP, 5 μg of myelin basic protein (MBP), and 5 μCi of [γ-^{32}P]ATP. After incubation at 30°C for 20 min, the kinase reaction was terminated by washing the beads with HEPES binding buffer. The phosphorylated proteins were eluted in 30 μl of 2× Laemmli sample buffer, resolved on an SDS–10% polyacrylamide gel, and then autoradiographed. The procedures for the JNK activity assay were the same as those for ERK, except that 1 μg of agarose-bound glutathione *S*-transferase (GST)–c-Jun(1-223) (19) and 5 μCi of [γ-^{32}P]ATP were added directly to the cell lysate for the kinase reaction. In some of the experiments, plasmids encoding hemagglutinin (HA)-JNK were transfected into BAEC and the exogenous epitope-tagged JNK was immunoprecipitated with mouse anti-HA monoclonal antibody (Boehringer Mannheim). The procedures used to assay the activity of the JNK were the same as those described above.

Plasmids. ERK(K52R) and ERK(K71R) were gifts from Melanie Cobb of the University of Texas. HA-JNK, Gal4–c-Jun(1 to 223), Gal4–c-Jun(1 to 223, Ala63/73), MEKK(K-M), Raf301, RasN17, mSOS1, and ΔmSOS1 were described previously (10, 14, 24, 32, 33, 39). To construct JNK(K-R), JNK1 was mutated in pBluescript by PCR to introduce an *Nco*I site at its first ATG codon and a point mutation at codon 55 which replaced the Lys-55 with an Arg. The mutations were confirmed by DNA sequencing. The mutated JNK1 was then subcloned into expression vector SRα3HA (9) at the *Nco*I and *Bgl*II sites to create JNK(K-R).

AP-1–TRE activation assays. Expression plasmids encoding the wild-type, active, or dominant negative forms of p21^{ras}, MEKK, and JNK were cotransfected with either 4xTRE-Pl-Luc or MCP1-Luc-540 into BAEC at 70% confluence by transient-transfection protocols. 4xTRE-Pl-Luc is a construct in which the rat prolactin promoter conjugated to the luciferase reporter is driven by four copies of the TRE consensus sequence, and MCP1-Luc-540 is a construct in which the luciferase reporter is driven by the 540-bp MCP-1 promoter (41). The pSV-β-galactosidase plasmid, which contains a β-galactosidase gene driven by a simian virus 40 promoter and enhancer, was also included in the cotransfection to monitor transfection efficiency. After incubation for 6 h, the cells were washed with PBS and incubated with fresh medium for another 24 to 48 h to reach confluence. The cells in the tissue culture flasks were then seeded onto glass slides and used either for fluid shearing experiments or as static controls. The luciferase reporter activities in the various experiments, normalized for transfection efficiency, were used to assess the suppressing effects of the various negative mutants on the shear-induced transcription activation mediated by AP-1–TRE.

RESULTS

p21^{ras} is activated by fluid shearing in BAEC. To investigate whether fluid shearing leads to an activation of p21^{ras} in EC, i.e., an increased ratio of p21^{ras} · GTP to p21^{ras} · GDP, confluent monolayers of the ^{32}P-labeled BAEC were subjected to a fluid shearing of 12 dynes/cm^2 for various time periods and the cells were lysed and subjected to guanine nucleotide binding assays. In the static controls, p21^{ras} was exclusively in its GDP-bound inactive form (Fig. 1). After 1 min of shearing, the ratio of p21^{ras} · GTP to p21^{ras} · GDP increased markedly.

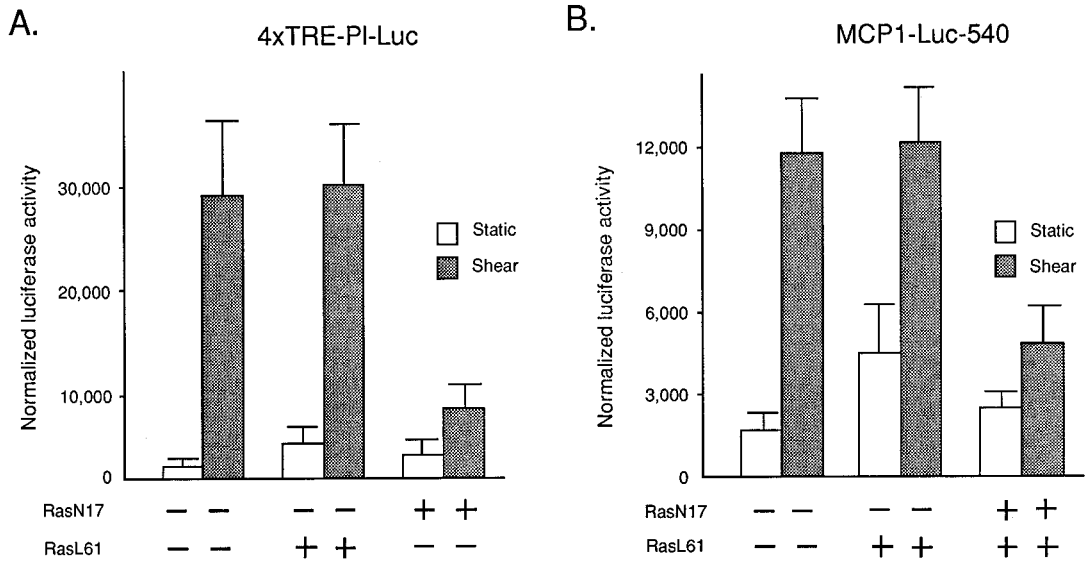

FIG. 2. RasN17 abolishes shear-induced AP-1–TRE activation. Empty vector pcDNA3, or an expression plasmid encoding RasL61 and/or RasN17, was cotransfected with 4xTRE-Pl-Luc or MCP1-Luc-540 into BAEC in a tissue culture flask. The DNA-transfected cells were reseeded on culture slides to confluence and either subjected to fluid shearing of 12 dynes/cm^2 for 8 h or kept as static controls and then subjected to luciferase activity assays. The normalized luciferase activities are the luminometer readings of luciferase activity normalized for transfection efficiency on the basis of β-galactosidase activity. The results represent the means plus standard deviations from at least three experiments.

Densitometric analysis indicated that 17% ± 4% of all the guanine nucleotides bound to p21^{ras} was GTP bound. This GTP-bound active form gradually returned to the GDP-bound form afterwards. By 5 min after the beginning of shearing, the GTP-bound form decreased to 7% ± 3%. By 10 min, all p21^{ras} became inactive, as in the static controls. Thus, fluid shearing, like other extracellular stimuli, such as mitogens, cytokines, osmotic shock, and UV irradiation, induces a transient activation of p21^{ras}.

The shear-activated AP-1–TRE is mediated through p21^{ras}. By using transient-transfection assays, we have previously shown that the luciferase reporters driven by TRE (i.e., 4xTRE-Pl-Luc and MCP1-Luc-540) can be induced by fluid shearing. Transactivation assays using c-Jun or c-Jun–c-Fos expression plasmids also induced these TRE-driven constructs (41), indicating that the transcription factor that mediates such activation is AP-1. To investigate whether Ras is upstream of the shear-induced activation of AP-1–TRE, we examined the effects of RasN17 on the induction of 4xTRE-Pl-Luc and MCP1-Luc-540. RasN17 is a dominant negative mutant of Ras in which Ser-17 in the wild type has been replaced by Asn so that the affinity to GTP is dramatically reduced (14). As shown in Fig. 2, in the plasmid control experiments, fluid shearing caused 23- and 6.5-fold increases in induction (the luciferase activities in the sheared cells compared with those in the static controls) of 4xTRE-Pl-Luc and MCP1-Luc-540, respectively. We also transfected the expression plasmid encoding RasL61, the active form of p21^{ras} in which the Gln-61 in the wild type has been replaced by Leu, into BAEC. The expression of RasL61 increased the basal level of expression of both 4xTRE-Pl-Luc and MCP1-Luc-540. However, the induction by fluid shearing was not affected. In contrast, the cotransfection of RasN17 with either 4xTRE-Pl-Luc or MCP1-Luc-540 into BAEC significantly decreased the shear-induced luciferase reporter activities. These results, together with those presented in Fig. 1, suggest that functional p21^{ras} is required for AP-1–TRE-mediated gene expression in response to fluid shearing.

Fluid shearing activates JNK to a greater extent than ERK. We have shown that in EC, ERK-1 and ERK-2 are phosphorylated by fluid shearing (41). To investigate whether the phosphorylation of ERK led to their increased kinase activities, BAEC were subjected to shearing at 12 dynes/cm^2 for various lengths of time. As shown in Fig. 3A, such a mechanical stimulation induced a rapid activation of ERK to cause MBP phos-

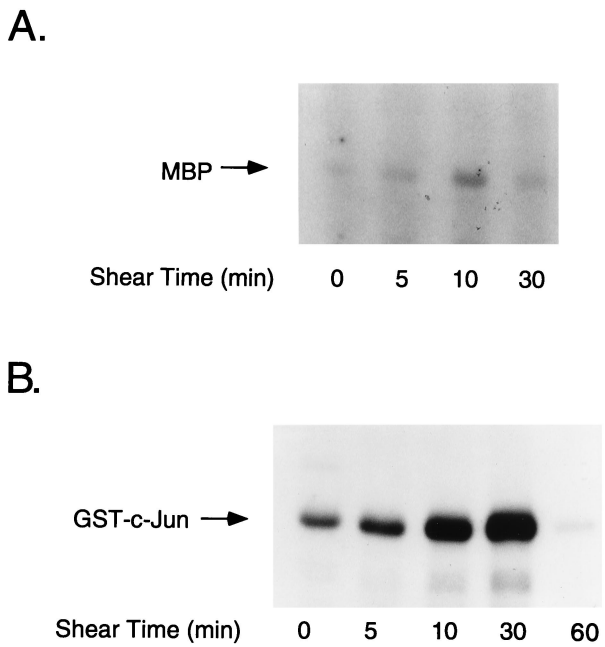

FIG. 3. Fluid shearing preferentially activates JNK. BAEC were subjected to fluid shearing of 12 dynes/cm^2 for the lengths of time indicated. (A) After shearing, ERK was immunoprecipitated and a kinase activity assay was performed in the presence of MBP and [γ-^{32}P]ATP. (B) The cell lysate was incubated with agarose-bound GST–c-Jun to precipitate JNK, and then [γ-^{32}P]ATP was added. Static controls are represented by time zero results, and the 20- and 39-kDa phosphorylated MBP and GST–c-Jun are indicated. Densitometric analysis indicated that the factors by which peak induction exceeded those in the static controls were 1.8 at 10 min (A) and 10.5 at 30 min (B).

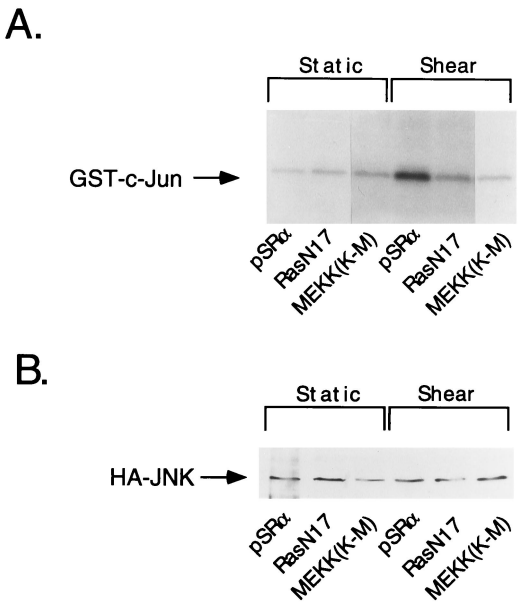

FIG. 4. $p21^{ras}$ and MEKK are upstream of shear-activated JNK. Ten micrograms of expression plasmid encoding HA-JNK was cotransfected with either 10 μg of pSRα empty vector, RasN17, or MEKK(K-M) into BAEC in a T-75 tissue culture flask. The transfected cells on culture slides were either kept as static controls or subjected to a fluid shearing of 12 dynes/cm^2 for 30 min. After shearing, HA-JNK was immunoprecipitated with anti-HA monoclonal antibody and subjected to kinase assays using GST–c-Jun and [γ-^{32}P]ATP as substrates. The phosphorylated GST-c-Jun is indicated (A). RasN17 and MEKK(K-M) inhibited the shear-induced phosphorylation of GST–c-Jun. (B) Immunoblotting of the cellular proteins with anti-HA monoclonal antibody. The equal intensities of the bands recognized by enhanced chemiluminescence detection reagents indicate that the amounts of expressed HA-JNK in different samples were the same.

phorylation, which peaked at 10 min and decreased afterwards. Densitometric analysis indicated that the peak activity was 1.8-fold greater than that in the static controls. To investigate whether fluid shearing activates JNK as it does ERK, agarose-bound GST–c-Jun, a fusion protein containing GST and the N-terminal moiety (1 to 223) of c-Jun, and [γ-^{32}P]ATP were added to the cell lysate for a JNK kinase reaction. As shown in Fig. 3B, fluid shearing induced a rapid activation of JNK in BAEC to cause c-Jun phosphorylation, which peaked at 30 min and decreased afterwards. After the cells had been exposed to the shearing for 60 min, the JNK activities returned to a level lower than that in the static controls. The peak JNK activity at 30 min, determined by densitometry, was 10.5-fold greater than that in the static cells. Kinase assay results for static cells incubated with fresh medium or with conditioned medium collected from sheared cells showed no difference in kinase activities from the results for the static controls (data not shown). These results indicate that the activation of cytoplasmic kinases in the sheared cells was attributable to the action of mechanical force rather than to the medium supplements or to the metabolites released from the cells during shearing. Furthermore, fluid shearing of BAEC activates JNK to a greater extent and for a longer duration than ERK.

$p21^{ras}$ and MEKK are upstream of shear-activated JNK. We further investigated whether $p21^{ras}$ is upstream of shear-activated JNK. Kinase assay results shown in Fig. 4 indicate that the exogenous epitope-tagged HA-JNK was also activated by fluid shearing in the transfected BAEC. The cotransfection of RasN17 inhibited the shear-induced kinase activity of HA-JNK, as manifested by the phosphorylation of its substrate, GST–c-Jun (Fig. 4A). It has been shown that the expression of Ras activates MEKK (27) and that the induction of MEKK stimulates JNK (48). Cotransfection of the catalytically inactive enzyme MEKK(K-M), in which the Lys-432 had been replaced by a Met (33), with HA-JNK also reduced shear-induced JNK activity (Fig. 4A). These results indicate that fluid shearing activates a Ras-MEKK-JNK pathway in vascular EC.

Negative mutants of MEKK and JNK block shear-activated AP-1–TRE. To further examine whether MEKK mediates the shear-induced reporter driven by AP-1–TRE, we cotransfected MEKK(K-M) with either 4xTRE-Pl-Luc or MCP1-Luc-540. In addition, JNK(K-R), a kinase-deficient JNK1 in which the Lys-52 in the wild type was replaced by an Arg, was constructed. If the Ras-MEKK-JNK pathway is upstream of AP-1–TRE, the use of either MEKK(K-M) or JNK(K-R) to block the functions of the wild types should attenuate shear-induced AP-1–TRE. Figure 5A indicates that the cotransfection of expression plasmids encoding MEKK(K-M) or JNK(K-R) did reduce the factor by which shear-induced 4xTRE-Pl-Luc activity exceeded static controls from 21.5 to 12.5 and 4, respectively. Cotransfection of these catalytically inactive mutants with MCP1-Luc-540 also reduced the shear-induced luciferase reporter activities (Fig. 5B). In contrast, cotransfection of the expression plasmids encoding wild-type MEKK or JNK did not affect shear-induced 4xTRE-P1-Luc and MCP1-Luc-540 activities (data not shown). Thus, MEKK and JNK are upstream of AP-1–TRE-mediated gene expression in response to shear stress.

Raf301, ERK(52R), and ERK(71R) have little effect on shear-activated AP-1–TRE. In response to stimulation by growth factors or phorbol ester, the Ras-ERK pathway is activated (11, 46), leading to the activation of AP-1–TRE (16). Raf-1 contributes directly to ERK activation in this pathway but not to JNK activation (33). Mechanical shearing has a less potent effect on ERK than on JNK (Fig. 3), which seems to indicate that the Ras–Raf-1–ERK pathway is less important for downstream gene expression. To test the role played by the Ras–Raf-1–ERK pathway in the shear-induced activation of AP-1–TRE, we used dominant negative mutants of Raf-1 and ERK to block this pathway and examined AP-1–TRE-mediated reporter activities in response to mechanical stimulation. Raf301 is a dominant negative mutant of Raf-1 in which the Lys-375 in the wild type has been replaced by Trp (24). ERK(K71R) and ERK(K52R) are the dominant mutants of ERK-1 and ERK-2, in which the respective Lys-71 and Lys-52 in the wild types have been replaced by Arg (36). Figure 6 shows that cotransfection of Raf301 had little effect on shear-induced 4xTRE-Pl-Luc reporter activity. Similarly, neither ERK(71R), ERK(52R), nor a combination of these two ERK dominant negative mutants attenuated reporter activity in response to fluid shearing. Experiments using MCP1-Luc-540 also showed that none of these negative mutants was able to affect shear-induced luciferase activity (data not shown). Thus, the Ras–Raf-1–ERK pathway is not essential for the shear-induced activation of AP-1–TRE.

Fluid shearing increases c-Jun transcriptional activity. It seems that the induction of 4xTRE-Pl-Luc and MCP1-Luc-540 by fluid shearing results from an up-regulated c-Jun, which is activated by the Ras-MEKK-JNK pathway. To test whether fluid shearing increases the transcriptional activity of c-Jun, plasmid Gal4–c-Jun, encoding the fusion protein of the Gal4 DNA binding domain and the c-Jun activation domain (1 to 223), was cotransfected with 4xGal-Luc, a chimeric construct consisting of the Gal4 binding sequence and the luciferase reporter, into BAEC. Compared with that in the static controls, fluid shearing increased luciferase activity more than

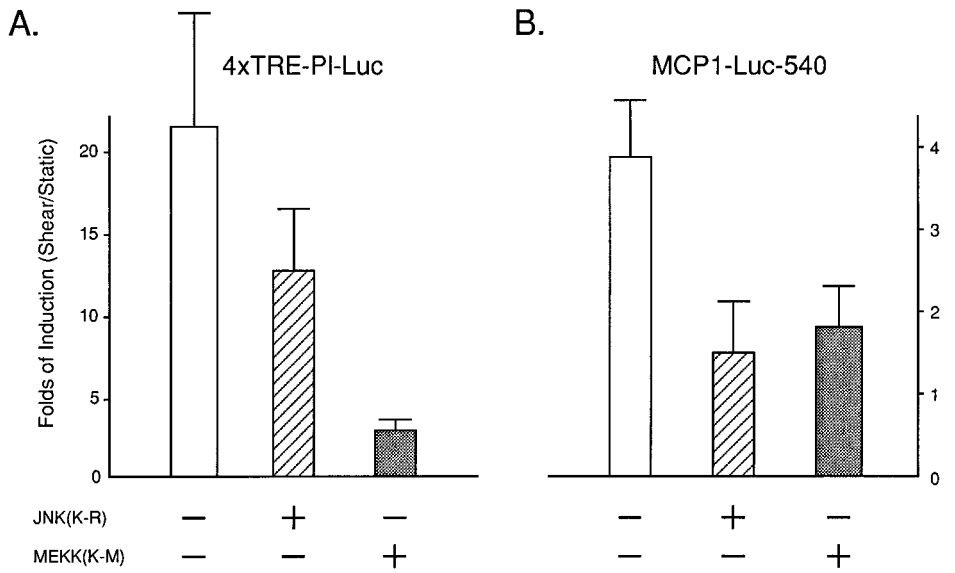

FIG. 5. JNK(K-R) and MEKK(K-M) attenuate shear-induced AP-1–TRE activation. Expression plasmids encoding JNK(K-R) or MEKK(K-M) were cotransfected with 4xTRE-Pl-Luc or with MCP1-Luc-540 into BAEC. The experimental conditions were the same as those described in the legend to Fig. 2. Induction was measured as the normalized luciferase activities in the experimental cells relative to those in the static controls. The results are the means plus standard deviations from six experiments.

fourfold in the sheared cells (Fig. 7), indicating increased c-Jun transcriptional activity. In contrast, the plasmid encoding the mutated Gal4–c-Jun, in which the phosphorylation sites Ser-63 and Ser-73 had been replaced by Ala, showed a marked reduction in response to fluid shearing. Furthermore, coexpression of RasN17 or MEKK(K-M) also attenuated such shear-induced transcriptional activity. Thus, the fluid shearing-induced activation of AP-1 is at least in part due to increased c-Jun transcriptional activity, which is in turn activated through the phosphorylation of Ser-63 and Ser-73 by the Ras-MEKK-JNK pathway.

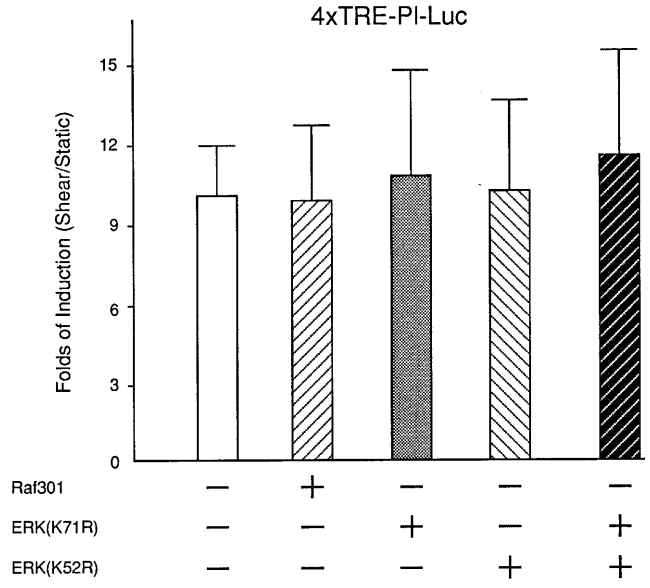

FIG. 6. Raf-1 and ERK are not required for shear-induced AP-1–TRE. Chimeric construct 4xTRE-Pl-Luc was transiently transfected into BAEC alone or with other plasmids encoding the dominant negative mutants Raf301, ERK (K71R), and ERK(K52R) for fluid shearing and luciferase assays. The experimental conditions were the same as those described in the legend to Fig. 2. Induction was measured as the normalized luciferase activities in the experimental cells relative to those in the static controls. The results are the means plus standard deviations from six experiments.

ΔmSOS1 attenuates shear-induced c-Jun transcriptional activity. Son of sevenless (Sos) is a guanine nucleotide exchange factor that activates $p21^{ras}$ by converting the GDP-bound inactive state to the GTP-bound active state (4, 13). To explore whether Sos is a upstream molecule regulating shear-activated Ras signaling, expression plasmids encoding ΔmSOS1, a dominant negative mutant of mouse SOS1 in which the guanine nucleotide exchange domain has been deleted (39), were cotransfected with Gal4–c-Jun and 4xGal-Luc into BAEC. The transfected cells were then subjected to fluid shearing and then luciferase assays. As shown in Fig. 7B, ΔmSOS1 attenuated shear-induced c-Jun transcriptional activity. The inducibility by fluid shearing in cells transfected with mSOS1, the wild-type mouse SOS1, was comparable to that in cells transfected with empty vectors.

DISCUSSION

The results of this study provide several lines of evidence that $p21^{ras}$ plays critical roles in the responses of vascular EC to fluid shearing. First, the guanine nucleotide exchange on Ras, i.e., the conversion of Ras · GDP to Ras · GTP, was promoted by fluid shearing. Second, the dominant negative mutant of $p21^{ras}$, RasN17, inhibited the shear-induced signal transduction pathway, including JNK and its downstream c-Jun transcriptional activity. Third, RasN17 also abrogated the reporter activities of 4xTRE-Luc and MCP1-Luc-540, two chimeric constructs whose induction by fluid shearing is mediated by AP-1–TRE (41). It is not known where and how mechanical stimuli are transduced to biochemical signals. Since $p21^{ras}$ is a membrane-associated protein and its activation of downstream Raf-1 and MEKK is through direct interactions on the membrane (27, 28, 36, 38, 43), our results suggest that the mechanical-to-biochemical transmitting process occurs at least in part

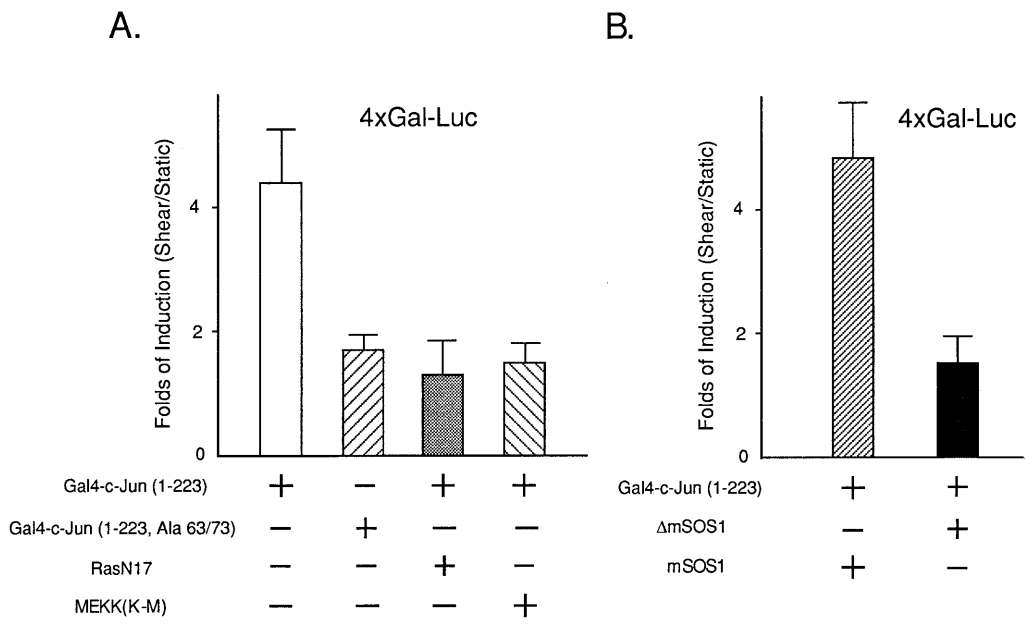

FIG. 7. Fluid shearing increases the transcriptional activity of c-Jun, and appropriate mutants attenuate this shear-induced activity. (A) BAEC were transfected with 6 μg of 4xGal-Luc and 6 μg of Gal4–c-Jun(1-223), 6 μg of Gal4–c-Jun(1-223, Ala63/73), 18 μg of RasN17, and/or 18 μg of MEKK(K-M), as indicated. (B) BAEC were transfected with the same amounts of 4xGal-Luc and Gal4–c-Jun(1-223) as for panel A together with either 18 μg of either mSOS1 or ΔmSOS1. The experimental conditions were the same as those described in the legend to Fig. 2. Induction was measured as the normalized luciferase activities in the experimental cells relative to those in the static controls. The results are the means plus standard deviations from six experiments.

on the cellular membrane. Wang et al. (47) suggest that the integrins on the basal membrane constitute a mechanoreceptor and that stress fibers are necessary to transmit the applied forces. Similarly, Davies et al. (8) suggest that focal adhesion complexes at the abluminal endothelial membrane are mechanically responsive elements coupled to the cytoskeleton. These suggestions imply that the signals initiated by fluid shearing originate from the abluminal side of EC. In contrast, the burst production of nitric oxide is dependent upon the activation of G proteins (25), which are located at the luminal surface.

Ras can activate both the ERK and JNK pathways (27, 33). The signaling in response to growth factors such as epidermal growth factor (EGF) and nerve growth factor is mediated through both the Ras-ERK and Ras-JNK pathways in PC12, MRC5, and HeLa cells (33, 34). In contrast, inflammation-related cytokines (e.g., tumor necrosis factor and interleukin-1), environmental stresses (e.g., osmotic pressure), and UV irradiation selectively activate the Ras-JNK pathway but not the ERK pathway (17, 19, 26, 33, 42, 44). Mechanical shearing is a form of force borne by vascular EC and many other cell types, such as osteoblasts, under physiological conditions. The application of such physiological forces to static cells cultured in flow chambers provides a sudden change of hemodynamic environment. This in vitro system mimics the pathophysiological changes during reperfusion after flow stoppage. We demonstrate here that fluid shearing of vascular EC increased the activation of JNK by more than 10-fold and increased the activation of ERK by a much lower magnitude (1.8-fold) and for a shorter duration (Fig. 3). These results are in good agreement with several reports presented recently at the American Heart Association's 68th Scientific Sessions. Morooka et al. demonstrated that reperfusion of ischemic kidney induced a rapid activation of JNK (35). Bogoyevitch et al. reported that reperfusion of rat heart induced a 10- to 50-fold increase in the activation of JNK but not of ERK (2). Besides confirming the weak activation of ERK, we demonstrated that the blockade of the $p21^{ras}$-Raf-ERK pathway by Raf301, K71R, or K52R did not affect TRE-mediated reporter activities in response to fluid shearing (Fig. 6). In contrast, the blockade of the $p21^{ras}$-MEKK-JNK pathway by either MEKK(K-M) or JNK(K-R) significantly attenuated the shear-induced reporter activities (Fig. 5). MEKK(K-M) and JNK(K-R), with mutations at the ATP binding sites in which the conserved Lys is replaced by either a Met or an Arg, act like dominant negative mutants of MEKK and JNK, respectively (33, 43a). Thus, the induction of MEKK-JNK by fluid shearing plays a key role in the activation of the downstream AP-1–TRE, an effect which is probably mediated through c-Jun. On the other hand, the activation of c-Fos, which is dependent on ERK-$p62^{TCF}$ (9), seems to be not necessary for such an activation. It appears that the Jun-Jun homodimer, rather than the Jun-Fos heterodimer, serves as the activator in this shear-elicited signal transduction pathway. Presumably, the shear-induced activation of JNK by JNK kinase phosphorylates the preexisting, latent c-Jun in the cytoplasm, and this is followed by the translocation of the activated c-Jun into the nucleus, where the Jun-Jun homodimer activates the target gene by interacting with TRE. Transactivation assays using RSV-Jun, an expression plasmid encoding c-Jun, showed effects similar to those of fluid shearing in inducing MCP1-Luc-540 (41). These results, taken together, suggest that the $p21^{ras}$-MEKK-JNK kinase-JNK pathway is necessary and sufficient to activate AP-1–TRE-mediated gene expression in EC in response to fluid shearing (Fig. 8). In addition to the phosphorylation of the preexisting c-Jun, the induction of AP-1 activity may occur at the transcriptional level. Mechanical shearing induces c-*jun* mRNA, which remains at an elevated plateau level for at least 4 h (21). It is not known whether the activated JNK in the sheared cells would activate the de novo-synthesized c-Jun through the phosphorylation of Ser-63 and

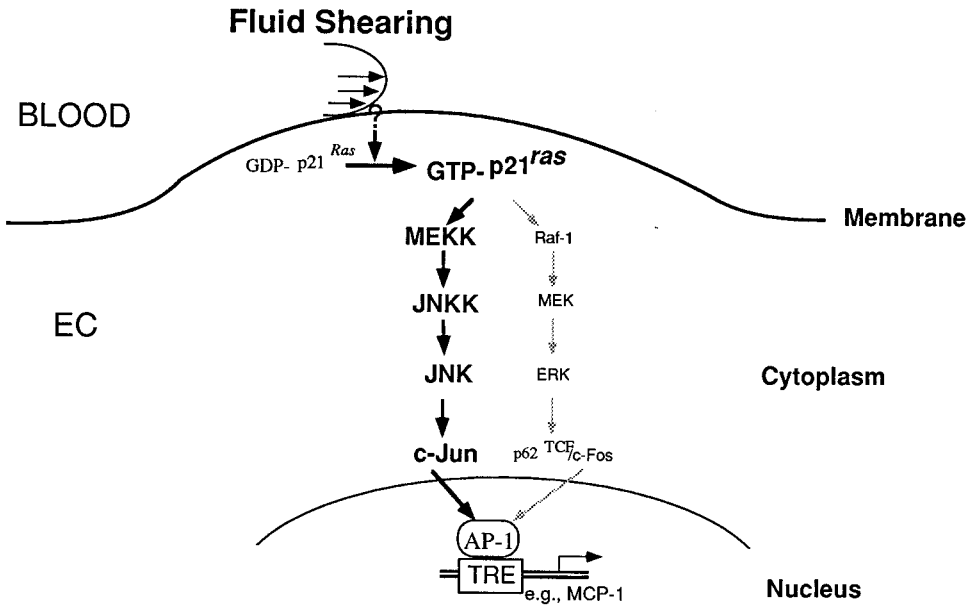

FIG. 8. The fluid shearing-elicited signal transduction pathways leading to AP-1–TRE-mediated gene expression (as exemplified by the MCP-1 gene) in EC. The mechanical-to-biochemical transduction most likely occurs on the membrane by undefined sensors or receptors that activate membrane-associated p21^{ras}. Subsequently, the MEKK-JNK kinase-JNK pathway is activated preferentially. As a result, c-Jun is phosphorylated to increase its transcriptional activity and the Jun-Jun homodimer activates the TRE-containing promoters. The Raf-MEK-ERK pathway, although also activated by the hemodynamic force, is much less important and probably not necessary for the activation of AP-1–TRE.

Ser-73 (34) and, if it did, what the duration over which the activated c-Jun homodimer could activate the downstream genes would be.

The duration of MAPK activation by different extracellular stimuli may determine whether the cells can elicit differentiation or proliferation responses (31). In PC12 cells, ERK activation is sustained for several hours following nerve growth factor stimulation, thus leading to differentiation of these cells into sympathetic neurons. In contrast, the response is transient after EGF stimulation and the result is proliferation rather than differentiation (45). Whether the activation is sustained or transient is dependent on the receptor tyrosine kinases (RTKs) which activate p21^{ras}. The cellular responses to fluid shearing, including the activation of p21^{ras}, JNK, and the downstream IE genes (e.g., the MCP-1 gene), are all transient and rapid. The cells are conceivably desensitized by the applied mechanical force following the initial activation. We have previously found that preshearing desensitizes EC against further 12-*O*-tetradecanoylphorbol-13-acetate-induced ERK phosphorylation (41). Such mechanically induced transient responses and desensitization have their physiological significance in vascular EC. Serving as the barrier between blood and vessel wall, these cells need a desensitization mechanism to protect them from the continuous stimulation imposed by hemodynamic forces. In bends and bifurcations, where the shearing forces are low and the blood flow is disturbed, there may not be the same degree of desensitization as in the lesion-resistant areas, where the endothelium is subjected to a relatively constant laminar flow with high shearing forces. When cumulated over years, the small differences in the mechanical environments (i.e., the magnitude of shearing forces and flow pattern) of the cells in these different regions may have considerable pathophysiological consequences.

It is intriguing that Sos can be upstream of the fluid shearing-activated p21^{ras}. We have recently found that the negative mutants of Grb2 and Sos can also partially block shear-induced 4xTRE-Pl-Luc and MCP1-Luc-540 activities in BAEC (23). Grb2 is an adapter protein which contains one src homology 2 domain and two src homology 3 domains. Grb2 binds to Sos, a guanine nucleotide exchange factor specific to p21^{ras}. Thus, the upstream mechanisms by which mechanical-to-biochemical transduction activates the Ras pathway may be similar to those for growth factor stimuli. It remains to be investigated how common upstream signals diverge to activate JNK (mechanical stimuli) and ERK (growth factor).

In summary, the p21^{ras}–MEKK–JNK–AP-1–TRE pathway provides a molecular mechanism for signal transduction in endothelial responses to mechanical stimulation. It would be interesting to investigate whether RTKs are the mechanical force receptors or sensors on the membrane that execute mechanical-to-biochemical transduction to activate such a pathway. The similarity between fluid shearing and EGF in inducing endothelial responses, including the involvement of molecules containing an src homology 2 domain, such as Grb2, suggests that RTKs, especially the EGF receptor subfamily, may play an important role in transducing the mechanical stimuli into biochemical signals. Recently, it has been shown that the small GTP-binding proteins Rac and Cdc42 are upstream of JNK (6, 32). Constitutively activated Rac and Cdc42 stimulate the catalytic activity of JNK (32). Dominant negative mutants of Rac and Cdc42 effectively reduce EGF-activated JNK (6). Thus, Rac and Cdc42 are crucial intermediates in the signaling pathway leading from activated RTKs to JNK. These findings reinforce the hypothesis that RTKs are candidates for the mechanical force sensor, but direct experimental evidence is still lacking. Whatever the sensor mechanism, the p21^{ras}–MEKK–JNK–AP-1–TRE pathway seems to be part of a coordinated programming, which includes other possible components, such as G proteins, Ca^{2+}, and integrins, etc. The synergism and/or cross-communication among these different signaling pathways probably plays significant roles in constituting the endothelial responses to hemodynamic forces.

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