Rapid Activation of Ras by Fluid Flow Is Mediated by $G\alpha_q$ and $G\beta\gamma$ Subunits of Heterotrimeric G Proteins in Human Endothelial Cells

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Objective—Temporal gradients in fluid shear stress have been shown to induce a proatherogenic phenotype in endothelial cells. The biomechanical mechanism(s) that enables the endothelium to respond to fluid shear stress requires rapid activation and signal transduction. The small G protein Ras has been identified as an early link between rapid mechanotransduction events and the effects of shear stress on downstream signal-transduction cascades. The aim of this study was to elucidate the upstream mechanotransduction signaling events mediating the rapid activation of Ras by fluid shear stress in human endothelial cells.

Methods and Results—Direct measurement of Ras-bound GTP and GDP showed that fluid-flow activation of Ras was rapid (10-fold within 5 seconds) and dose dependent on shear stress magnitude. Treatment with protein tyrosine kinase inhibitors or pertussis toxin did not significantly affect flow-induced Ras activation. However, activation was inhibited by transient transfection with antisense to $G\alpha_q$ or the $G\beta\gamma$ scavenger β-adrenergic receptor kinase carboxy terminus. Transfection with several $G\beta\gamma$ subunit isoforms revealed flow-induced Ras activation was most effectively enhanced by $G\beta_{1\gamma2}$.

Conclusions—These results suggest that the rapid, shear-induced activation of Ras is mediated by $G\alpha_q$ through the activity of $G\beta\gamma$ subunits in human vascular endothelial cells. **(*Arterioscler Thromb Vasc Biol*. 2003;23:994-1000.)**

Key Words: endothelium ■ Ras ■ G proteins ■ shear stress

Atherosclerosis remains a leading cause of morbidity and mortality in the Western world. The formation of atherosclerotic lesions in the vasculature does not occur in a random fashion.[1] Hemodynamic forces interacting with an active vascular endothelium have long been implicated in the nonrandom localization of atherosclerotic lesions. Detailed analyses of fluid mechanics in atherosclerosis-susceptible vascular regions reveal a strong correlation between endothelial cell dysfunction and rapid changes in hemodynamic shear stress (or from flow recirculation and oscillation).[1] In vitro, rapid changes in fluid shear stress (generated by the sudden onset of flow) have been shown to induce a proatherogenic phenotype in endothelial cells.[2–4] The biomechanical mechanism(s) that enables the endothelium to respond to rapid changes in fluid shear stress would require virtually instantaneous activation to accommodate such a rapid stimulus. Given that the endothelial membrane is directly exposed to fluid shear stress, the lipid membrane itself is likely to act as, or harbor the primary components of, the shear-sensing mechanism. The activation of membrane-bound G proteins has recently been recognized as a mediator of flow-induced endothelial response.[4–6] G proteins are 1 of the earliest known shear-responsive cellular elements. The activation of $G\alpha_q/\alpha_{11}$ and $G\alpha_{i\text{-}3}/\alpha_o$ protein subunits occurs within 1 second of flow onset.[7] When G proteins are isolated and reconstituted in a phospholipid bilayer in the absence of cytoskeletal elements or other receptors, $G\alpha_q$ and G_i respond specifically to fluid shear stress.[8] Cellular localization and rapid activation strongly implicate G proteins as a primary sensor of hemodynamic forces. However, the early mechanochemical signaling events transducing proatherosclerotic mechanical stimuli into biochemical signals that link G-protein activation and downstream signaling pathways are not fully understood.

The mechanochemical mechanism(s) of fluid shear–induced signal transduction is thought to be mediated through the Ras/mitogen-activated protein kinase (MAPK) pathway.[9] Ras mediates the effects of receptor and nonreceptor tyrosine kinases in mitogenic signaling pathways and regulates G protein–dependent activation of extracellular signal–regulated kinase (ERK) and *N*-terminal Jun kinase (JNK).[10,11] Shear-induced Ras activation of the ERK and JNK pathways can be inhibited by the tyrosine kinase inhibitors genistein

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and herbimycin A.[10,12] Whereas Ras is a mediator of shear-induced activation for both ERK and JNK, activation of each pathway differs in its time course, force dependence, and heterotrimeric G-protein subunit selectivity as upstream regulators. For example, shear-dependent Ras activation of ERK in bovine arterial endothelial cells (BAECs) is mediated by $G\alpha_{i\text{-}2}$, whereas JNK activation is dependent on a $G\beta\gamma$ subunit.[10] However, in fetal BAECs, shear stress activation of ERK does not involve $G\alpha_{i\text{-}2}$,[13] and direct measurements of G-protein activation in human umbilical vein endothelial cells (HUVECs) have shown $G\alpha_q$ and $G\alpha_{i\text{-}3}$ to be activated by fluid flow, whereas fluid-shear activation of $G\alpha_{i\text{-}2}$ was not detected.[12] Furthermore, shear-dependent activation of heterotrimeric G-protein signaling pathways in endothelial cells occurs by either a pertussis toxin (PTx)–sensitive or –insensitive pathway.[6,10] In BAECs, fluid shear stress differentially regulates JNK and ERK activation by PTx-insensitive G protein–dependent and $G\alpha_{i\text{-}2}$-dependent pathways, respectively.[10] Thus, it appears that in different experimental models of the endothelial shear stress response, shear-induced Ras/MAPK activation can be regulated through different heterotrimeric G protein–dependent mechanisms, and >1 type of G-protein subunit can be activated by fluid shear stress.

The present study examined the mechanisms of shear-induced mechanochemical signal transduction leading to the proatherosclerotic activation of the MAPK pathway by fluid shear stress in HUVECs. The kinetics of Ras activation by fluid flow and the involvement of different G-protein subunits were investigated. Results showed a rapid, fluid shear–induced Ras activation mediated by $G\alpha_q$ and $G\beta\gamma$in HUVECs.

Methods

Cell Culture and Shear Stress

Primary HUVECs were harvested from fresh umbilical cords as previously described.[14] Cells (1×10^6) were seeded onto glass slides (Fisher Scientific) and then maintained at 37°C, 5% CO_2/air, in ATP-free medium 199 (Hyclone) with 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin sulfate, and 20% fetal calf serum (Hyclone). Six hours before the experiment, growth medium was replaced with serum-free medium (ATP-free medium 199 supplemented with 2% bovine serum albumin).

In a conventional parallel-plate flow chamber,[6] confluent HUVEC monolayers on glass slides were subjected to fluid flow at 1, 3, 10, or 20 dynes/cm^2 of steady shear stress. Onset of flow was sudden. Duration of flow varied between protocols (5 seconds to 60 minutes). Serum-free medium was used as the perfusing medium for all experimental procedures. Perfusing medium was driven by a syringe pump (Harvard Apparatus). All flow chambers and accompanying apparatus were maintained at 37°C throughout the experiment. Time-matched sham controls were performed for all experimental groups. At the end of a given duration of flow stimulation, cells designated for determination of shear-induced proliferation were quickly removed from the chamber and incubated at 37°C in medium 199–bromodeoxyuridine (10 fmol/L BrdU[15]) for 22 hours. Detection of BrdU incorporation into cellular DNA during DNA synthesis was determined by using a commercially available in situ monoclonal antibody kit (Boehringer Mannheim). All other cells were snap-frozen on dry ice. In some experiments, high-viscosity perfusion medium (serum-free medium supplemented with 2.8% [wt/vol] dextran to give a viscosity of 3.8 cP) was used to apply shear stress to the cells.

Plasmid Constructs and Transfection

Full-length antisense $G\alpha_q$ vector ($G\alpha_q$ AS) was a gift of Nuoki Mochizuki (International Medical Center, Toyama, Japan); a vector expressing β-adrenergic receptor kinase carboxy terminus (βARK-ct) fused to CD8 in pcDNA3[16] was provided by S. Gutkind (National Institute of Dental Research, Bethesda, Md); and vectors for $G\alpha_q$ wild type ($G\alpha_q$ WT), a constitutively active $G\alpha_q$ ($G\alpha_q$ QL), $G\beta_1$, $G\beta_2$, $G\gamma_1$, and $G\gamma_2$ were gifts of M. Simon (California Institute of Technology, Pasadena, Calif). Endotoxin-free DNA was prepared by using plasmid DNA preparation kits and following the manufacturer's instructions (Qiagen).

HUVECs were transfected by using Superfect Reagent (Qiagen). In brief, preformed DNA/Superfect complexes were added to 80% confluent cells for 3 hours in the presence of medium 199 containing 2% fetal calf serum. The amount of DNA added per slide, 3 μg, was kept constant. Cells were used for experiments 2 days after transfection. Transfection efficiency was determined to be 40% to 50% by assaying for the reporter gene β-galactosidase with use of a chemiluminescent substrate (Galacton) as recommended by the supplier (Tropix).

Measuring Ras-Bound GTP and GDP

Ras-bound GTP and GDP were measured as previously described.[17] In brief, shear-exposed cells on glass slides were rinsed with phosphate-buffered saline and then snap-frozen at −70°C. Frozen cells were extracted in ice-cold RIPA buffer (50 mmol/L HEPES, pH 7.4; 10 mmol/L $MgCl_2$; 150 mmol/L NaCl; 1% Nonidet P-40; 0.5 mmol/L phenylmethylsulfonyl fluoride; and 2 μg/mL each of aprotinin, leupeptin, and pepstatin) by shaking for 5 minutes at 4°C. The cell extracts were centrifuged at 10 000*g* for 3 minutes. Supernatants were divided in half, and either 3 μg of the anti-Ras antibody Y13[hyphen]259 (Santa Cruz Biotechnology) or 3 μg of rat IgG (Cappel) was added. To both samples, goat anti-rat IgG and protein G–agarose (Santa Cruz Biotechnology) were added and supplemented with NaCl, sodium dodecyl sulfate (SDS), and deoxycholate to final concentrations of 500 mmol/L, 0.05%, and 0.5%, respectively. The samples were shaken gently for 1 hour at 4°C, and the immunoprecipitates were washed 4 times in RIPA with 500 mmol/L NaCl, 0.05% SDS, and 0.5% deoxycholate and 2 times in 20 mmol/L trisphosphate. Immunoprecipitates were resuspended in 30 μL of 5 mmol/L trisphosphate, pH 7.4, 2 mmol/L dithiothreitol, and 2 mmol/L EDTA and heated at 100°C for 3 minutes. After the samples were cooled on ice, they were centrifuged at 10 000*g* for 5 minutes, and GDP and GTP in the supernatants were measured.

The amount of GTP eluted from immunoprecipitated Ras was determined by using a kinetic assay in which GTP is converted to ATP by nucleoside 5′-diphosphate kinase in the presence of excess ADP, and ATP is consumed by the firefly luciferase reaction to produce light. The reaction was monitored with a photon-counting luminometer. Levels of GTP in anti-Ras–immunoprecipitated samples were determined by integrating photon counts over 10 minutes and subtracting counts obtained from a paired rat IgG control sample. The amount of GDP was determined by equilibrium conversion of GDP to GTP with pyruvate kinase and phosphoenolpyruvate and the GTP measured as described previously. The amounts of GTP and GDP in the samples (in fmol nucleotide per mg of cell lysate protein) were determined from standard curves prepared with each set of samples. Protein amounts in cell lysates were determined by a Bradford assay (Bio-Rad) with bovine serum albumin as a standard. Ras-bound GTP divided by Ras-bound GDP plus GTP was used as an index of Ras activation.

Western Blotting

Cells were lysed in a buffer of 30 mmol/L Tris, pH 7.4, 150 mmol/L EDTA, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, and 1 mmol/L sodium orthovanadate for 20 minutes at 4°C. Cell lysates were centrifuged to remove insoluble material, and proteins were denatured by boiling in Laemmli sample buffer containing 100 mmol/L dithiothreitol. Equal amounts of protein from each cell lysate were resolved by SDS–polyacrylamide gel electrophoresis

(12%), electroblotted onto a polyvinylidine difluoride membrane (Immobilon-P, Millipore), and probed with antibodies specific to $G\alpha_q$ (Santa Cruz Biotechnology) or endothelial growth factor receptor (EGFR) and active EGFR (Transduction Labs). Goat anti-rabbit (or anti-mouse) IgG conjugated to horseradish peroxidase was used as a secondary antibody, and staining was visualized by an enhanced chemiluminescent detection method (Pierce) according to the manufacturer's instructions.

Statistics

Data are expressed as mean±SD from at least 3 independent experiments. Data were first evaluated by ANOVA, followed by the Student-Newman-Keuls test for multiple comparisons, with $P<0.05$ considered statistically significant.

Results

Fluid Shear Stress Rapidly and Transiently Activates Ras in HUVECs

Measurement of Ras-bound GTP and GDP in HUVECs was performed to determine the time and dose dependence of fluid flow–induced Ras activation. The kinetics of Ras activation were examined at 20 dyne/cm^2 (Figure 1A). Activation was rapid and transient, reaching a maximum of ≈10-fold over stationary controls at 5 seconds, the earliest time point, then declining to ≈4-fold after 15 minutes of steady fluid flow. A similar pattern was observed in HUVEC proliferation. HUVEC proliferation was 180±12% (n=5) of stationary controls at 5 seconds but was not significantly different between 20 minutes of steady fluid flow and stationary controls (n=8; data not shown). When HUVECs were exposed to 10 seconds of flow, Ras was activated in an intensity-dependent manner, monotonically increasing through 0, 1, 3, 10, and 20 dyne/cm^2 (Figure 1B). As a positive control, Ras activation was measured after stimulation with EGF (50 ng/mL for 5 minutes) or phorbol ester (200 nmol/L for 1 hour) and was found to be ≈10- and 15-fold, respectively, of untreated samples (Figure 1C).

To determine whether fluid flow–induced Ras activation was influenced by mass-transport effects, a high-viscosity perfusion medium was used to increase shear stress while maintaining a low flow rate. HUVECs were subjected to a shear rate of 100 s^{-1} and a shear stress of either 1 dyne/cm^2 (standard medium) or 3.8 dyne/cm^2 (high-viscosity medium) for 10 seconds. At the constant shear rate, Ras activation increased with increased shear stress and was approximately equal in magnitude to the activation measured after standard perfusion medium at 3 dyne/cm^2 of shear stress (Figure 1B). Similarly, to test whether the hydrostatic pressure used to drive the fluid flow was contributing to the cellular response, hydrostatic pressure in the chamber was increased without permitting fluid flow. The outlet of the chamber was closed and the perfusion reservoir raised to a height that would have produced a shear stress of 10 dynes/cm^2 (17.8 mm Hg).

Ras Activation by Fluid Shear Stress Is Insensitive to Tyrosine Kinase Inhibition

To determine whether tyrosine kinases were involved in the shear-induced activation of Ras, HUVECs were treated with tyrosine kinase inhibitors before exposure to fluid shear of 20 dyne/cm^2 for 10 seconds. Neither herbimycin A (1 μg/mL for 12 hours) nor genistein (100 μmol/L for 6 hours) significantly inhibited Ras activation by fluid flow (Figure 2A). To ensure that the inhibitors were functional under the experimental conditions, Western blots of HUVECs treated with the inhibitors and stimulated with EGF (50 ng/mL for 5 minutes) were probed for both activated and total EGFR (Figure 2B). Both herbimycin A and genistein inhibited EGFR activation.

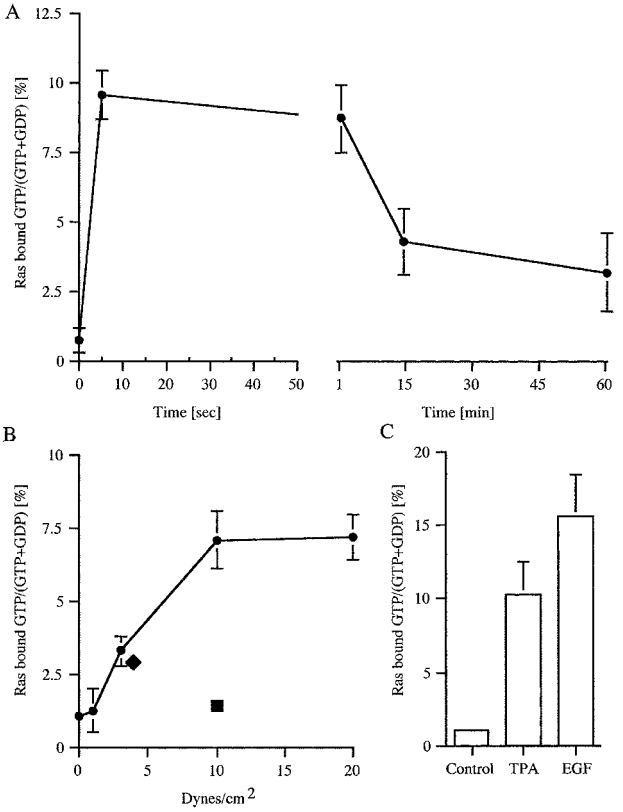

Figure 1. Fluid shear stress rapidly and transiently activates Ras in HUVECs. Ras-bound GTP divided by Ras-bound GDP plus GTP was used as an index of Ras activation. A, HUVECs were exposed to fluid shear stress of 20 dyne/cm^2 for the times indicated. B, HUVECs were exposed to fluid shear stress of 0, 1, 3, 10, and 20 dyne/cm^2 for 10 seconds. Controls: ◆, HUVECs exposed to 10 seconds of flow at a shear rate of 100/s with a high-viscosity (3.8 cP) medium, giving a shear stress of 3.8 dyne/cm^2; ■, HUVECs subjected to a hydrostatic pressure of 17 mm Hg (equal to pressure during flow that generated 10 dyne/cm^2 shear stress) without flow for 10 seconds. C, HUVECs were treated with phorbol ester (200 nmol/L for 1 hour) or EGF (50 ng/mL for 5 minutes) as positive controls for Ras activation. Data are expressed as the percentage of Ras-bound GTP divided by total Ras-bound GDP plus GTP. Values represent mean±SD for 3 independent experiments.

Activation of Ras by Fluid Shear Stress Is Insensitive to Pertussis Toxin and Cholera Toxin

To elucidate the role of heterotrimeric G proteins in shear stress activation of Ras in human endothelial cells, HUVECs were treated with pertussis toxin (PTx; 1 mg/mL for 6 hours) or cholera toxin (CTx; 1 mg/mL for 6 hours) before exposure to fluid shear of 20 dyne/cm^2 for 10 seconds (Figure 2A). Neither PTx, an inhibitor of the $G\alpha_i$ class of G proteins, nor CTx, an activator of $G\alpha_s$, displayed a significant affect on shear-induced activation of Ras.

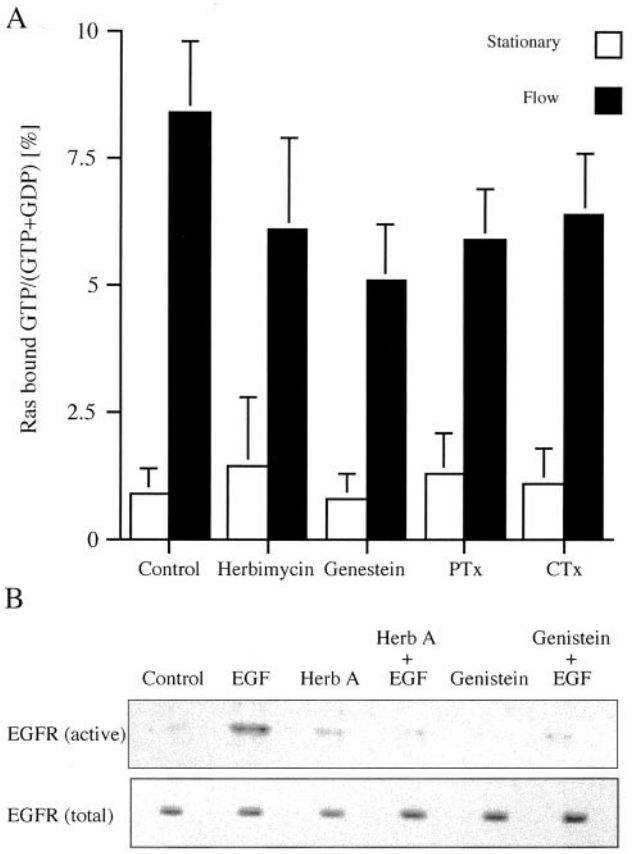

Figure 2. Protein tyrosine kinase inhibitors and PTx did not affect Ras activation by fluid shear stress. A, HUVECs pretreated with PTx (1 mg/mL for 6 hours), CTx (1 mg/mL for 6 hours), genistein (100 μmol/L for 6 hours), or herbimycin A (1 μg/mL for 12 hours) were exposed to fluid shear stress of 20 dyne/cm^2 for 10 seconds. Ras activation is expressed as the percentage of Ras-bound GTP divided by Ras-bound GDP plus GTP. Values represent mean±SD for 3 independent experiments. No significant differences of flow minus stationary Ras activation exist among the treatment groups (power of 0.78 for 75% inhibition). B, Western blot of HUVECs treated with or without EGF, genistein, or herbimycin A under the same conditions as in A and probed for both total EGFR and activated EGFR.

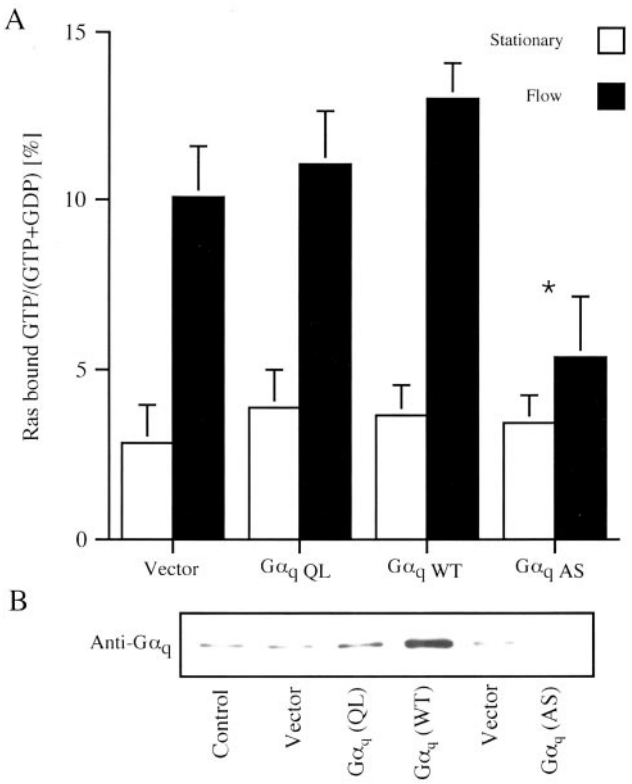

Figure 3. Activation of Ras by fluid shear stress was inhibited by transient transfection with antisense Gα_q. HUVECs were transiently transfected with a vector containing wild-type Gα_q (WT), constitutively active Gα_q (QL), or antisense Gα_q (AS) or with an empty vector (3 μg DNA per slide in each case) and exposed to fluid shear stress of 20 dyne/cm^2 for 10 seconds. Ras activation is expressed as the percentage of Ras-bound GTP divided by Ras-bound GDP plus GTP after normalization to efficacy of transfection. Values represent mean±SD for 3 to 6 independent experiments. *$P<0.01$ vs empty vector (flow minus stationary values). No significant difference was found between empty vector and Gα_q (WT) or Gα_q (QL). B, Equal amounts of cell lysate proteins were analyzed by Western blotting with an anti-Gα_q antibody. A representative immunostain is shown.

Antisense Inhibition of Gα_q Blocks Ras Activation by Fluid Shear Stress

The role of the PTx-insensitive Gα_q subunit in shear-induced Ras activation was investigated by transient transfection of HUVECs with constructs for wild-type Gα_q (WT), constitutively active Gα_q (QL), or antisense Gα_q (AS). Gα_q expression levels in transfected cultures were analyzed by Western immunoblotting (Figure 3B). Transfected cultures were subjected to fluid shear of 20 dyne/cm^2 for 10 seconds and analyzed by comparing the percentage of Ras-bound GTP in flow-stimulated minus stationary cultures (Figure 3B). Transfection with antisense Gα_q inhibited shear activation of Ras by 74% compared with that seen in empty vector–transfected controls ($P<0.01$). Wild-type transfected cultures displayed a small and nonsignificant increase (29%) in shear stress activation of Ras. Transfection with Gα_q, a constitutively active Gα_q unable to bind G$\beta\gamma$ subunits, did not result in significant increases in Ras activation under stationary or flow conditions.

Involvement of G$\beta\gamma$ Subunits in Ras Activation by Fluid Shear Stress

The role of G$\beta\gamma$ subunits in the activation of Ras by fluid shear stress was assessed by transfection of HUVECs with the G$\beta\gamma$ scavenger βARK-ct. Transfected cultures were either stimulated by fluid shear of 20 dyne/cm^2 for 10 seconds or treated with EGF (50 ng/mL) for 5 minutes and analyzed by comparing the percentage of Ras-bound GTP in stimulated minus stationary (untreated) cultures (Figure 4). βARK-ct expression inhibited shear-induced Ras activation by 73% compared with that seen in empty vector–transfected controls ($P<0.01$). Although EGF-induced Ras activation was decreased by transfection with βARK-ct, this inhibition was not statistically significant (power of $\approx$0.70 for 75% inhibition, equivalent to the inhibition of shear-induced Ras activation by βARK-ct or Gα_q transfection).

To determine which specific G$\beta\gamma$ subunits were involved in the activation of Ras by fluid shear stress, HUVECs transfected with $\beta_{1\gamma1}$, $\beta_{1\gamma2}$, $\beta_{2\gamma1}$, or $\beta_{2\gamma2}$ subunits were exposed to fluid shear of 20 dyne/cm^2 for 10 seconds (Figure 5).

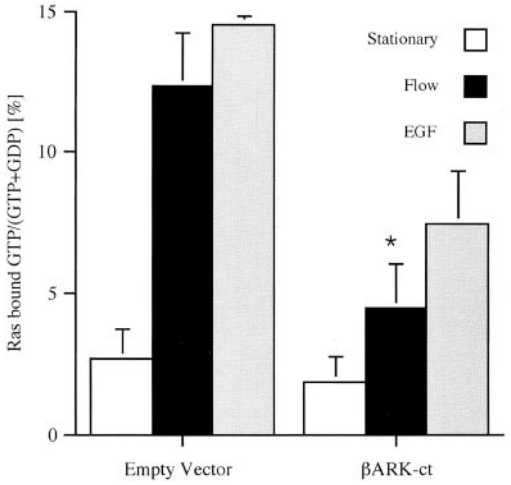

Figure 4. Activation of Ras by fluid shear stress was inhibited by transient expression of βARK-ct. HUVECs were transiently transfected with a vector containing βARK-ct or an empty vector and exposed to fluid shear stress of 20 dyne/cm^2 for 10 seconds or treated with EGF (50 ng/mL) for 5 minutes. Ras activation is expressed as the percentage of Ras-bound GTP divided by Ras-bound GDP plus GTP after normalization to efficacy of transfection. Values represent mean±SD for 3 or 5 independent experiments. *$P<0.01$ vs empty vector (flow minus stationary values). βARK-ct transfection did not have a significant effect on EGF stimulation of Ras (power of ≈0.70 for 75% inhibition).

HUVECs transfected with $\beta_{1\gamma2}$ displayed the greatest enhancement of shear-induced Ras activation (80% increase over flow-stimulated empty vector–transfected controls; $P<0.01$), whereas transfection with $\beta_{1\gamma1}$ caused a 55% increase ($P<0.01$).

Discussion

This study has presented several lines of evidence that heterotrimeric G proteins mediate the rapid activation of Ras by fluid shear stress in human endothelial cells. Careful attention was paid to determining the specificity of the Ras activation by fluid shear stress. Dose-dependent shear stress activation was found to be independent of mass-transport and hydrostatic pressure effects (Figure 1B). When the viscosity of the perfusion medium was increased while maintaining a constant perfusion rate (thus, increasing shear stress without changes in flow rate), Ras activation was approximately equal in magnitude to the activation measured with standard perfusion medium at varying flow rates. These observations demonstrated that fluid flow–induced Ras activation was driven by shear stress rather than flow rate. Previous studies have also reported that the exposure of human vascular endothelial cells to sustained hydrostatic pressure might stimulate a proliferative response.[18] To test whether the hydrostatic pressure used to drive the fluid flow in the current study was contributing to the cellular response, hydrostatic pressure in the chamber was increased without permitting fluid flow. This procedure also failed to stimulate Ras activation.

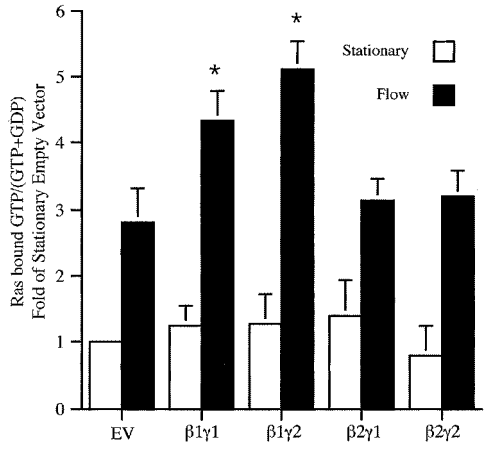

Figure 5. Gβγ composition affects enhancement of Ras activation by fluid shear stress. HUVECs were transiently transfected with $\beta_{1\gamma1}$, $\beta_{1\gamma2}$, $\beta_{2\gamma1}$, $\beta_{2\gamma2}$, or empty vector and exposed to shear stress of 20 dyne/cm^2 for 10 seconds. Results are normalized to transfection efficiency and expressed as fold increase over non–flow-stimulated cells transfected with empty vector. Values represent mean±SD for 3 or 5 independent experiments. *$P<0.01$ vs empty vector, $\beta_{2\gamma1}$, and $\beta_{2\gamma2}$.

All fluid flow profiles used throughout this study contained a significant temporal gradient, resulting from the sudden onset of flow, followed by steady fluid shear stress of varying duration. A temporal gradient in shear stress is defined as the localized change in shear stress over a small period of time at any given point. Large temporal gradients in fluid shear due to the change of shear direction have been linked to the pathogenesis of atherosclerosis[19] and intimal hyperplasia.[20] Temporal gradients in fluid shear stress have been shown to stimulate distinct, proatherosclerotic biochemical pathways in human endothelial monolayers,[2–4] whereas prolonged steady fluid flow has been shown to be atheroprotective and to stimulate opposing biochemical pathways.[9]

In BAECs, it has been shown that the sudden onset of fluid flow at 12 dyne/cm^2 activates Ras within 1 minute[21] in an Src-dependent manner.[22] In the present study, Ras activation was rapid in response to the sudden onset of fluid flow. Ras activation peaked at the earliest time point measured (5 seconds) but was significantly reduced from peak activation (though still elevated above baseline) after 1 hour of steady flow (Figure 1A). The reduction in Ras activation after extended exposure to steady flow is consistent with the atheroprotective role of steady shear stress. Chen et al[23] have reported that 5 minutes of shear stress induces a transient tyrosine phosphorylation of Flk-1 and concomitant association of Flk-1 with the adapter protein Shc. The authors hypothesized that Ras is subsequently activated by the resulting Shc-Grb2-mSOS complex. The finding of this study, that shear stress–induced Ras activation was maximal by no later than 5 seconds after onset of flow, contradicts this hypothesis, because events occurring after 5 minutes of shear stimulation (tyrosine phosphorylation of Flk-1) cannot be responsible for observations made at 5 seconds. The present study also found that the protein tyrosine kinase inhibitors genistein and herbimycin A did not inhibit Ras activation by fluid flow (Figure 2). This suggests that blockade of shear-induced MAPK activation by these tyrosine kinase inhibitors seen in other studies was due to an activity downstream of Ras. Therefore, the tyrosine phosphorylation of receptor tyrosine kinases (RTKs) after flow stimulation probably occurs after

Ras activation, possibly through signaling pathway cross-talk[24] or as the result of a parallel mechanotransduction pathway with slower kinetics than the G protein–mediated mechanism. Although a direct link between Ras activation and proatherosclerotic endothelial proliferation was not shown in this study, rates of HUVEC proliferation followed a similar pattern to the shear-induced Ras activation time course (data not shown).

Ras activation plays a critical role in mitogenic signal transduction by G protein–coupled receptors (GPCRs).[25] MAPKs link external signals to nuclear responses, such as gene expression in response to growth factor binding to either RTK or GPCR.[26,27] The action of Ras in MAPK signal transduction is triggered by activation of $G\alpha_q$- or $G\alpha_i$-coupled GPCRs.[25,28] Stimulation of various GPCRs, such as those for lysophosphatidic acid,[29] thyroid-releasing hormone,[30] endothelin-1,[31] and bradykinin,[32] rapidly induce tyrosine phosphorylation of Shc and formation of Shc-Grb2 complexes, steps that couple both GPCR and RTK to Ras activation.[33] Activation of the Ras/MAPK pathway mediated by GPCR was reported to involve Src kinase.[25,34,35] It has also been demonstrated that $G\alpha_q$- and $G\alpha_i$-coupled receptors mediate stimulation of EGFR tyrosine phosphorylation and MAPK activation.[36]

Fluid shear stimulation of endothelial cells activates PTx-sensitive and -insensitive G proteins by an unidentified mechanism of mechanoreceptor.[6,7,10] Neither the inhibition of the $G\alpha_i$ class of G proteins with PTx nor stimulation of $G\alpha_S$ with CTx (Figure 2A) displayed a significant effect on shear-induced activation of Ras. This suggests that activation of Ras by fluid shear stress is not mediated by either $G\alpha_S$ or the $G\alpha_i$ class of G proteins in HUVECs. Significant inhibition of shear-induced Ras activation in human endothelial cells after transfection with antisense $G\alpha_q$ does implicate $G\alpha_q$-containing G proteins as the primary mediators of this response (Figure 3). In cardiac myocytes, $G\alpha_q$ signaling is sufficient to activate Ras,[26] and activation of Shc, Grb2, and mSOS, upstream of Ras, plays essential roles in $G\alpha_q$-mediated MAPK activation.[34] However, transfection of HUVECs with constitutively active $G\alpha_q$ was unable to bind $G\beta\gamma$ and did not induce Ras activation or affect its shear stress–stimulated response (Figure 3), thus suggesting that $G\alpha_q$-containing G proteins are involved in the activation of Ras by fluid shear stress but that their effect is not carried out by the activated $G\alpha_q$ subunit.

In a study by Jo et al[10] using BAECs, scavenging of free $G\beta\gamma$ by transfection with βARK-ct showed that shear stress activation of JNK is mediated by $G\beta\gamma$. The authors also found Ras is involved in the pathway. In that study, application of the same $G\beta\gamma$-scavenging technique significantly inhibited shear-induced Ras activation, demonstrating that the rapid activation of Ras was mediated by $G\beta\gamma$ subunits in human endothelial cells (Figure 4). A variety of mechanisms have been proposed for $G\beta\gamma$-mediated Ras activation in different systems. The $G\beta\gamma$ subunit, by virtue of its ability to interact with certain pleckstrin homology domains, might influence the activity of either mSOS or Ras-GEF, both of which have pleckstrin homology domains.[37] $G\beta\gamma$ generated by stimulation of muscarinic receptors activates ERK2 via a Ras-dependent pathway, possibly involving Shc.[28] Phosphatidyl inositol 3 kinase-γ, which is activated by $G\beta\gamma$,[38] has been implicated in $G\alpha_i$-mediated MAPK activation.[39] Phosphatidyl inositol 3 kinase-γ has also been shown to mediate shear stress–dependent activation of JNK.[40] However, the specific mechanism by which $G\beta\gamma$ activates Ras in fluid flow–stimulated endothelial cells remains to be clarified.

In addition to tissue-specific expression of receptors, G proteins, and effectors, there are important protein-protein interactions of $G\alpha$ and $G\beta\gamma$ subunit isoforms that determine biochemical specificity and cell type–specific responses to a given stimulus. $G\beta_{1\gamma1/2}$ is a more efficacious activator of phospholipase C-β_3 than is $G\beta_{5\gamma2}$[41], and $G\beta_{1\gamma2}$ is the most effective $G\beta\gamma$ dimer in phospholipase C-ϵ activation.[42] Of the $G\beta\gamma$ combinations examined in this study, overexpression of $G\beta_{1\gamma2}$ or $G\beta_{1\gamma1}$ generated significant enhancement of fluid flow–induced Ras activation, suggesting that these subunits are specific for this mechanotransduction pathway (Figure 5). Although these findings indicate a role for the $G\alpha_q$ and $G\beta\gamma$ subunit isoforms in the rapid shear–induced activation of Ras, it is unclear whether the $G\alpha_q$ and $G\beta\gamma$ subunit isoforms interact within the same heterotrimeric complex.

In summary, we have shown that rapid activation of Ras in human endothelial cells by temporal gradients in shear stress is mediated by $G\beta\gamma$ subunits dissociated from flow-activated $G\alpha_q$. Maximal Ras activation in response to fluid shear stress was rapid and occurred within 5 seconds. Transfection of HUVECs with antisense $G\alpha_q$ resulted in significant reduction of Ras activation by temporal gradients in shear stress, suggesting that the $G\alpha_q$ protein class mediated the response. However, transfection of a constitutively active $G\alpha_q$, which was unable to bind $G\beta\gamma$, did not induce Ras activation under stationary conditions, nor did it enhance the flow-induced response, suggesting that $G\beta\gamma$ subunits dissociated from the activated $G\alpha_q$ were mediating the Ras activation. This was confirmed by significant blockade of shear-induced Ras activation by temporal gradients in shear stress after transfection with the $G\beta\gamma$ scavenger βARK-ct. Taken together, these results suggest that the shear-induced MAPK activation pathway begins with shear-induced $G\alpha_q$ activation and $G\beta\gamma$-mediated stimulation of Ras. When considering these findings, it is important to bear in mind that the hemodynamic role in the localization and progression of atherosclerosis is an early event in the pathogenesis of the disease. Atherosclerosis is a protracted and multifactorial disease that involves many circulating blood elements, hemodynamic forces, and a complex cascade of molecular events within the endothelium and the arterial wall. The present study was solely designed to better elucidate the early signaling events transducing proatherosclerotic mechanical stimuli into biochemical signals.

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