Gab1, SHP2, and Protein Kinase A Are Crucial for the Activation of the Endothelial NO Synthase by Fluid Shear Stress

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Abstract—Fluid shear stress enhances NO production in endothelial cells by a mechanism involving the activation of the phosphatidylinositol 3-kinase and the phosphorylation of the endothelial NO synthase (eNOS). We investigated the role of the scaffolding protein Gab1 and the tyrosine phosphatase SHP2 in this signal transduction cascade in cultured and native endothelial cells. Fluid shear stress elicited the phosphorylation and activation of Akt and eNOS as well as the tyrosine phosphorylation of Gab1 and its association with the p85 subunit of phosphatidylinositol 3-kinase and SHP2. Overexpression of a Gab1 mutant lacking the pleckstrin homology domain abrogated the shear stress–induced phosphorylation of Akt but failed to affect the phosphorylation or activity of eNOS. The latter response, however, was sensitive to a protein kinase A (PKA) inhibitor. Mutation of Gab1 Tyr627 to phenylalanine (YF-Gab1) to prevent the binding of SHP2 completely prevented the shear stress–induced phosphorylation of eNOS, leaving the Akt response intact. A dominant-negative SHP2 mutant prevented the activation of PKA and phosphorylation of eNOS without affecting that of Akt. Moreover, shear stress elicited the formation of a signalosome complex including eNOS, Gab1, SHP2 and the catalytic subunit of PKA. In isolated murine carotid arteries, flow-induced vasodilatation was prevented by a PKA inhibitor as well as by overexpression of either the YF-Gab1 or the dominant-negative SHP2 mutant. Thus, the shear stress–induced activation of eNOS depends on Gab1 and SHP2, which, in turn, regulate the phosphorylation and activity of eNOS by a PKA-dependent but Akt-independent mechanism. **(*Circ Res*. 2005;97:1236-1244.)**

Key Words: Akt ■ blood flow ■ endothelial nitric oxide synthase ■ mechanotransduction ■ protein kinase A

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Although the endothelial NO synthase (eNOS) was initially described as a Ca^{2+}-dependent enzyme, it is now clear that the changes in the phosphorylation of several serine and threonine (and possibly also tyrosine) residues regulate NO production in response to Ca^{2+}-elevating agonists as well as to hemodynamic stimuli, such as cyclic stretch and fluid shear stress (for reviews, see Fleming and Busse[1] and Boo and Jo[2]). Most is known about the role played by Ser1177, which is situated in the reductase domain of the enzyme, and Thr495, which is situated in the calmodulin-binding domain, in the regulation of NO production. The phosphorylation of these sites appears to play a reciprocal role in the regulation of eNOS activity as Ser1177 becomes phosphorylated in response to endothelial cell activation, whereas Thr495 is constitutively phosphorylated but dephosphorylated on stimulation as a consequence of the activation of phosphatases.[3–5] The dephosphorylation of Thr495 facilitates the Ca^{2+}-dependent association of calmodulin with eNOS,[5] whereas the phosphorylation of eNOS on Ser1177 increases NO output in an apparently Ca^{2+}-independent manner.[6,7] As the maintained production of endothelium-derived NO in response to fluid shear stress is a Ca^{2+}-independent process,[8,9] it is generally assumed that the phosphorylation of eNOS on Ser1177 plays the predominant role in regulating eNOS activity in response to hemodynamic stimuli.

The kinases responsible for the phosphorylation of eNOS on Ser1177 vary with the stimuli applied and can be attributed not only to the activation of the Ca^{2+}/calmodulin-dependent kinase II in bradykinin-stimulated endothelial cells[5] but to Akt in cells stimulated with vascular endothelial growth factor[7] or hepatocyte growth factor (HGF)[10] and to Akt[6,7] and, probably more importantly, to protein kinase A (PKA)[11] in cells exposed to fluid shear stress. Relatively little is known about the molecular events that determine eNOS activity in response to fluid shear stress. It is generally assumed that fluid shear stress simultaneously initiates signaling within caveolae, focal adhesion sites and intercellular junctions (for review, see Davies et al[12]). We and others have

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recently reported that platelet-endothelial cell adhesion molecule-1 modulates endothelial cell activation in response to shear stress,[13,14] most probably by virtue of its ability to interact with signaling molecules such as the tyrosine phosphatase SHP2 (Src Homology 2-containing Protein tyrosine phosphatase 2) and the scaffolding protein Gab1 (Grb2-associated binder 1) (for recent reviews, see Newman and Newman[15] and Ilan and Madri[16]). Indeed, Gab-1 translocates from the cytoplasm to endothelial cell junctions in response to flow[17] and can associate with the p85 subunit of the phosphatidylinositol 3-kinase (PI3-K),[18,19] which is essential for the shear stress–induced phosphorylation of eNOS.[6]

In the present study, we determined the role of Gab1 and SHP2 in the shear stress–induced phosphorylation and activation of Akt and eNOS in cultured and native endothelial cells. We assessed the effect of a Gab1 mutant lacking the pleckstrin homology domain (ΔPHGab1) and compared its effects with those of a mutant in which Tyr627 was substituted with phenylalanine (YF-Gab1) to interfere with its interaction with SHP2,[19,20] as well as with a dominant-negative SHP2 mutant.

Materials and Methods

Materials

The phospho-specific antibodies recognizing eNOS Ser1177, phospho-Akt (Ser473), and Gab1 were from Cell Signaling (Munich, Germany). The antibody against SHP2 was from BD Biosciences (Heidelberg, Germany), the anti-p85 PI3-K antibody was from Upstate (Lake Placid, NY), the anti-eNOS and anti–phosphotyrosine antibodies were from Transduction Laboratories (Heidelberg, Germany), and the antibody against the catalytic subunit of PKA was from Santa Cruz Biotechnology (Heidelberg, Germany). HGF was purchased from R&D Biosystems (Abingdon, UK), the PKA inhibitor Rp-3′,5′-cyclic monophosphorothioate sodium salt (RpAMPS) was from Alexis Biochemicals (Grünberg, Germany), and H89 was from Calbiochem (Bad Soden, Germany). All the other substances were from Sigma (Munich, Germany).

Cell Culture and Shear Stress

Porcine aortic endothelial cells were isolated and cultured as described previously.[21] Because of the loss of several signaling components with time in culture, endothelial cells were used only after 1 passage. Confluent endothelial cells were washed twice with M-199 medium containing 0.1% BSA and after serum starvation for 4 hours were subjected to shear stress of 12 dynes/cm^2 in a cone-plate viscosimeter as described[22] or to HGF (50 ng/mL). In some experiments, the intracellular concentration of cGMP was measured in presence of the phosphodiesterase inhibitor (isobutyl methylxanthine, 0.1 mmol/L) using a specific radioimmunoassay (Amersham, Freiburg, Germany) as described[22] or PKA activity was assessed using a commercially available kit (Upstate) according to the instructions of the manufacturer.

Immunoprecipitation and Immunoblotting

Cells were lysed in a Triton X-100 lysis buffer, the immunoprecipitation of Gab1 was performed as previously described,[23] and proteins in the cell supernatants or immunoprecipitates were subjected to SDS-PAGE.[5] Proteins were detected using their respective antibodies and visualized by enhanced chemiluminescence using a commercially available kit (Amersham, Germany).

Preparation and Expression of Adenoviral Vectors

Details regarding the generation of the recombinant adenoviruses expressing WT-Gab1, a Gab1 mutant lacking the *N*-terminal pleckstrin homology domain (ΔPH-Gab1), and Y627F-Gab1 (YF-Gab1) as well as the dominant-negative SHP2 mutant (DSH2) are provided in the online data supplement available at http://circres.ahajournals.org.

For efficient expression of the recombinant proteins, confluent primary cultures of porcine aortic endothelial cells were infected with the respective adenovirus (3×10^6 pfu/mL). Four hours after infection in serum-free medium, cells were washed extensively in PBS and fresh medium containing 4% FCS was added and cells were incubated for a further 44 hours.

Adenoviral Infection of Mouse Carotid Arteries

Male C57BL/6 mice, 6 to 9 weeks of age (Charles River, Sulzfeld, Germany) were anesthetized with isoflurane. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The carotid arteries were perfused with saline solution and partially freed of connective tissue but maintained in situ. Virus solution (3×10^6 pfu in 20 μL) was introduced into the lumen before the arteries were ligated, excised, and placed in culture medium (MCDB 131 containing penicillin, 50 IU/mL streptomycin, 50 μg/mL, and 2% FCS) in a standard CO_2 incubator (37°C) for 4 hours. Thereafter, the ligatures were removed and the vessels washed extensively to remove the virus and then maintained overnight in culture medium. See the online data supplement for additional experimental details.

Flow-Induced Vasodilatation

Freshly isolated or adenovirus-treated mouse carotid arteries were cannulated and perfused in a video-monitored perfusion system (Living Systems Instruments, Burlington, Vt) as described.[24] Transluminal pressure was set at 80 mm Hg and vessels were constricted with phenylephrine (10 to 100 nmol/L) to approximately 80% of the baseline diameter. Once a stable contraction was achieved, flow was increased stepwise from 10 to 800 μL/min. Endothelium-dependent relaxation to acetylcholine (1 μmol/L) was assessed at the end of each experiment, and only vessels that responded with a dilatation of 60% or more of the phenylephrine-induced contraction were included in the study. Segments were then recovered, incubated with lysis buffer, and subjected to Western blotting to determine the effectiveness of the adenoviral infection.

Statistics

Data are expressed as the mean±SEM, and statistical evaluation was performed using Student's *t* test for unpaired data and 1-way ANOVA or ANOVA for repeated measures followed by Dunnett's post hoc test where appropriate. Values of $P<0.05$ were considered statistically significant.

Results

Effect of Shear Stress on the Tyrosine Phosphorylation of Gab1

A weak tyrosine phosphorylation of Gab1 was detected in confluent cultures of porcine aortic endothelial cells maintained under static conditions. However, exposure to fluid shear stress (12 dynes/cm^2) induced the rapid and marked tyrosine phosphorylation of Gab1 (Figure 1A). The tyrosine phosphorylation of Gab1 was evident as early as 2 minutes after stimulation and peaked after 30 minutes. Thereafter, tyrosine phosphorylation of Gab1 decreased slightly but remained elevated above control levels as long as shear stress was applied, ie, up to 24 hours. Phosphorylation of Gab1 was accompanied by its association with the protein tyrosine phosphatase, SHP2, and the p85 subunit of the PI3-K (Figure 1B).

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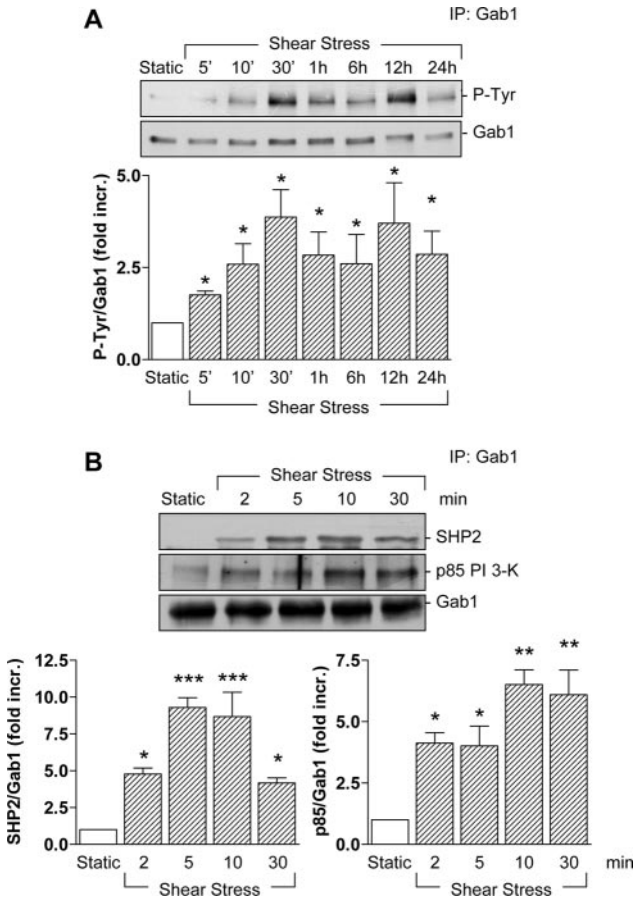

Figure 1. Effect of shear stress on tyrosine phosphorylation of Gab1 and its association with signaling molecules. Primary cultures of porcine aortic endothelial cells were either maintained under static conditions or exposed to fluid shear stress (12 dynes/cm^2) for up to 24 hours. A, Gab1 was immunoprecipitated and its tyrosine phosphorylation was assessed by Western blotting. Equal pull down of protein was confirmed by reprobing blots for Gab1. The bar graph summarizes data obtained in 4 independent experiments. *$P<0.05$ vs static conditions. B, Coimmunoprecipitation of the p85 subunit of the PI3-K and SHP2 with Gab1 from cells maintained under static conditions or exposed to shear stress. The blots shown are a representative of 3 independent experiments. incr. indicates increase.

Effect of a Gab1 Mutant Lacking the ΔPHGab1 on the Phosphorylation of Akt and eNOS in Endothelial Cells

Because exposure to fluid shear stress stimulated the association of Gab1 with the PI3-K, and a Gab1 PH domain decoy is reported to suppress the activity of a constitutively active Akt,[25] we assessed the consequences of the overexpression of the ΔPHGab1 mutant on the phosphorylation of Akt and eNOS in endothelial cells stimulated by either fluid shear stress or HGF, another stimulus for PI3-K and Akt activation.

Under static conditions the phosphorylation of Akt on Ser473 was low. Both fluid shear stress (12 dynes/cm^2) and HGF (50 ng/mL) elicited a pronounced increase in the phosphorylation of Akt in cells infected with a control virus (Figure 2A and 2B). However, only HGF was able to elicit the phosphorylation of Akt in cells expressing the ΔPHGab1 mutant (Figure 2B).

Because ΔPHGab1 blunted the shear stress–induced activation of Akt, we next determined its effect on the phosphorylation of eNOS. The phosphorylation of eNOS on Ser1177 was low in cells maintained under static conditions. In cells treated with a control virus, the phosphorylation of eNOS Ser1177 rapidly increased following the application of shear stress (Figure 2C) or the addition of HGF (Figure 2D). Overexpression of the ΔPHGab1 mutant failed to affect either the shear stress– or the HGF-induced phosphorylation of eNOS (Figure 2C and 2D).

Effect of the ΔPHGab1 Mutant on the Activation of eNOS

The ΔPHGab1 mutant attenuated basal NO production in unstimulated endothelial cells, whereas the overexpression of wild-type Gab1 increased basal NO production (supplementary Figure II). In cells treated with the control virus, shear stress elicited a 3.1±0.6-fold increase in cGMP levels. Despite the complete inhibition of the shear stress–induced activation of Akt, a similar increase (3.0±0.5-fold) in cGMP was detected in cells infected with the ΔPHGab1 mutant ($P<0.05$, n=4). Consistent with its lack of effect on the HGF-induced phosphorylation of Akt and eNOS, ΔPHGab1 did not affect the ability of HGF to increase endothelial NO production (supplementary Figure II).

As PKA can be activated by fluid shear stress and can also phosphorylate eNOS on Ser1177, we determined the effects of a PKA inhibitor on the shear stress–induced phosphorylation of eNOS on Ser1177. As reported above, the overexpression of ΔPHGab1 failed to affect the shear stress–induced phosphorylation of eNOS; however, the pretreatment of these cells with RpAMPS (10 μmol/L) prevented eNOS Ser1177 phosphorylation in cells expressing ΔPHGab1 (Figure 3A). These data indicate that although the ΔPHGab1 mutant completely prevented the shear stress–induced activation of Akt, the phosphorylation of eNOS on Ser1177 remained intact because of the activation of PKA. The shear stress–induced increase in the phosphorylation of eNOS Ser1177 was not observed in uninfected cells pretreated with the PKA inhibitors RpAMPS or H89 (10 μmol/L), whereas the ability of shear stress to induce the phosphorylation of Akt remained intact (Figure 3B and supplementary Figure III). The PKA inhibitors also prevented the shear stress–induced increase in endothelial GMP levels (Figure 3C).

In contrast to cells infected with the ΔPHGab1 mutant, the downregulation of Gab1 using an siRNA approach abrogated the shear stress–induced phosphorylation of both Akt and eNOS (supplementary Figure IV).

Effect of YF-Gab1 on the Shear Stress–Induced Activation of Akt and eNOS

Because we detected an increased association of Gab1 with SHP2 in response to shear stress (see Figure 1B), we next determined whether the association of these 2 proteins affects the shear stress–induced phosphorylation of eNOS. We reasoned that if the interaction between Gab1 and SHP2 is a prerequisite for the shear stress–induced activation of eNOS activation, the expression of a Gab1 mutant that is incapable of interacting with SHP2, ie, YF-Gab1,[20] may prevent the shear stress–induced activation of eNOS.

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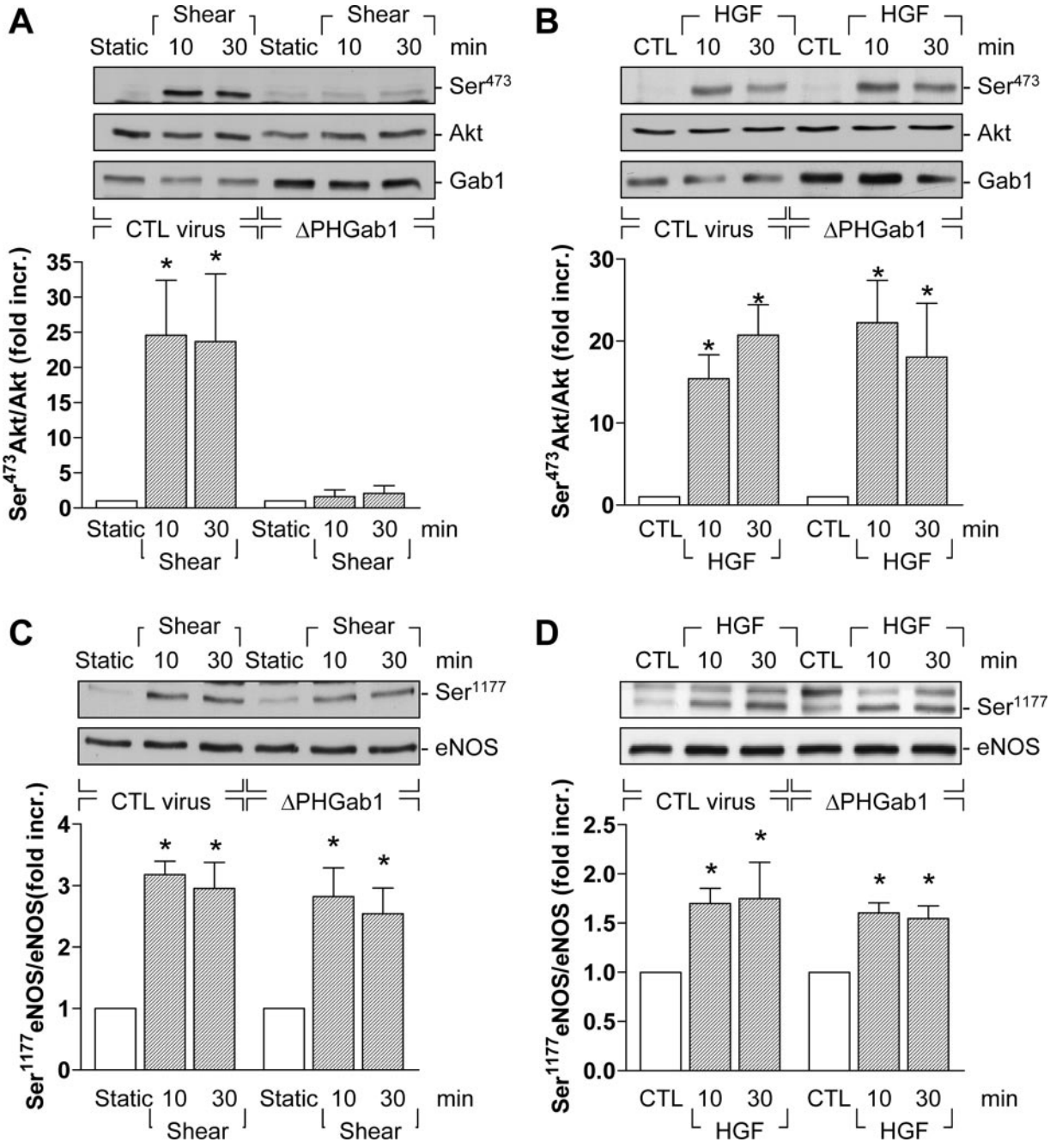

Figure 2. Effect of the overexpression of the ΔPHGab1 mutant on the shear stress– and HGF-induced phosphorylation of Akt and eNOS. Porcine aortic endothelial cells were infected with either control virus or virus encoding the ΔPHGab1 mutant 48 hours before stimulation. The phosphorylation of Akt on Ser473 or eNOS on Ser1177 were determined by SDS-PAGE followed by blotting with a phospho-specific antibody. Equal loading of each lane was confirmed by reprobing blots for total Akt or eNOS. Representative blots and bar graphs (4 independent experiments) summarizing the effects of ΔPHGab1 on the shear stress–induced (12 dynes/cm^2) (A and C) and HGF-induced (50 ng/mL) (B and D) phosphorylation of Akt (A and B) and eNOS (C and D). *$P<0.05$ vs static or control (CTL). incr. indicates increase.

The YF-Gab1 mutant failed to affect the HGF-induced phosphorylation of either Akt or eNOS (data not shown). However, exposure of endothelial cells overexpressing YF-Gab1 to shear stress prevented the phosphorylation of eNOS (Figure 4A and 4B), without affecting that of Akt (Figure 4A and 4C). Thus, although Gab1 appears to be necessary for the shear stress–induced activation of Akt through its N-terminal pleckstrin homology domain, its role in the shear stress–induced phosphorylation of eNOS is independent of Akt and dependent on its interaction with a protein, such as SHP2, that interacts with Gab1 at Tyr627.

Effect of a DSH2 on Responses to Shear Stress

The SHP2 mutant used expresses the tandem SH2 domains of SHP2 but lacks the catalytic domain and thus competes with endogenous SHP2 to act as a dominant-negative mutant. Infection of endothelial cells with the DSH2 viruses resulted in a significant overexpression of the protein without affect-

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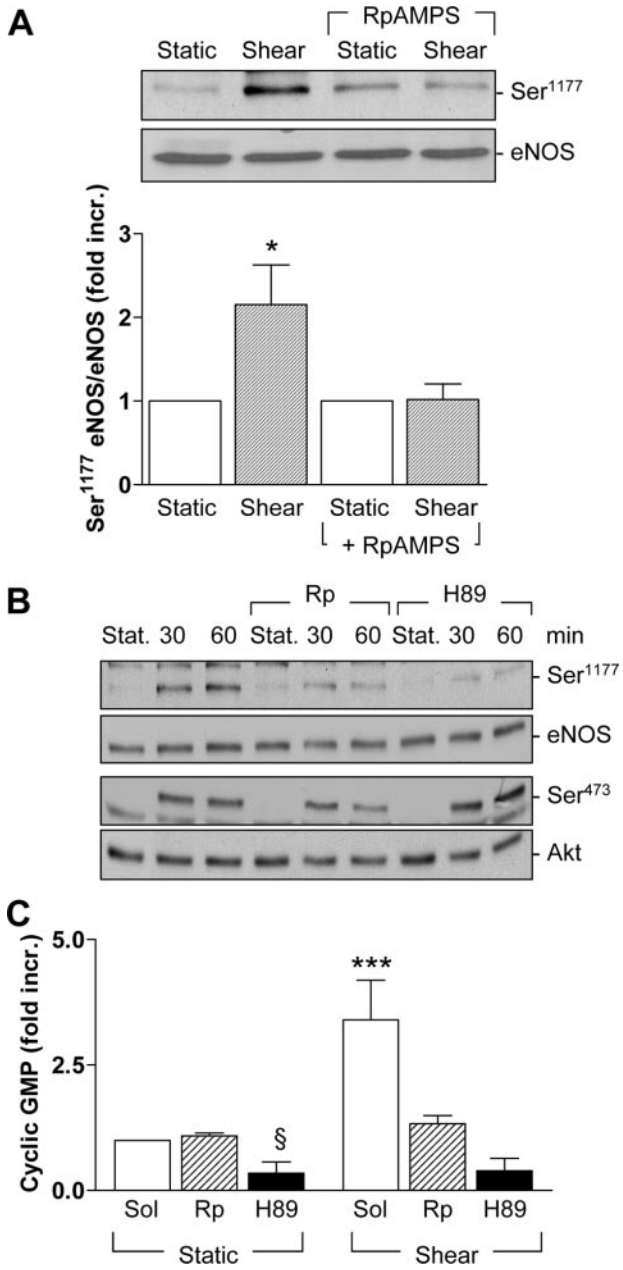

Figure 3. Effect of the ΔPHGab1 mutant and PKA inhibition on the response to shear stress. A, Confluent cultures of porcine aortic endothelial cells were infected with either empty vector or with adenovirus encoding the ΔPHGab1 mutant for 48 hours before stimulation. The phosphorylation of eNOS on Ser1177 was assessed in endothelial cells exposed to fluid shear stress (12 dynes/cm^2, 30 minutes) in the absence and presence of RpAMPS (10 μmol/L). B, Representative Western blots (3 independent experiments) showing the effect of RpAMPS and H-89 (10 μmol/L) on the shear stress–induced phosphorylation of eNOS and Akt. C, Effect of RpAMPS and H-89 on intracellular cGMP levels in endothelial cells maintained under static conditions or exposed to fluid shear stress for 30 minutes. The bar graphs summarize data from 3 to 5 independent experiments. *P<0.05, ***P<0.001 vs static conditions; §P<0.05 vs solvent (Sol). incr. indicates increase.

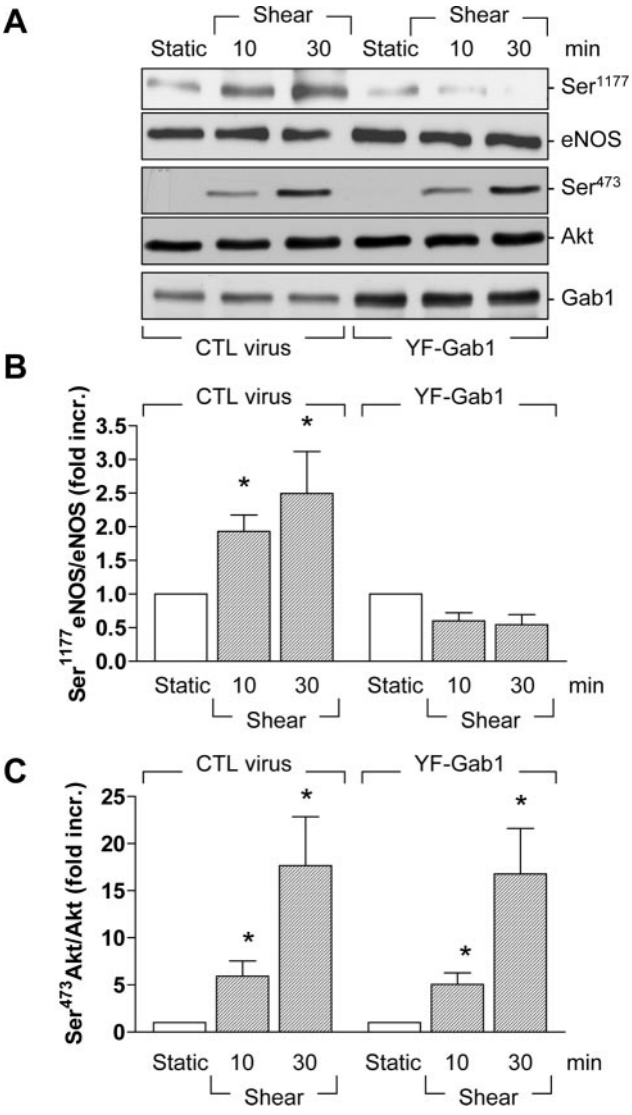

Figure 4. Effect of the YF-Gab1 mutant on the shear stress–induced phosphorylation of Akt and eNOS. Confluent cultures of porcine aortic endothelial cells were infected with either empty vector or with adenovirus encoding the Gab1YF mutant for 48 hours before stimulation with shear stress (12 dynes/cm^2). A, Representative Western blots showing the effect of the Gab1YF mutant on the phosphorylation of eNOS on Ser1177 and Akt on Ser473. The bar graphs (B and C) summarize data obtained in 4 independent experiments. *P<0.05 vs static conditions. incr. indicates increase.

ing endogenous levels of SHP2 (Figure 5A). Subsequent exposure of these cells to shear stress failed to induce the phosphorylation of eNOS, whereas the phosphorylation of Akt was unaffected (Figure 5). The shear stress–induced increase in cGMP was also attenuated in cells expressing the DSH2 protein. Levels of cGMP increased in response to shear stress by 2.8±0.2-fold (P<0.005, n=8) in cells infected with a control virus, whereas cGMP levels were only 91±14% (n=5, P=0.55) of basal levels in DHS2-expressing cells exposed to fluid shear stress.

Effect of Shear Stress on the Activity of PKA and Its Association With the eNOS Signalosome

As the PKA inhibitor RpAMPS, like the dominant-negative SHP2 mutant, prevented the shear stress–induced phosphorylation of eNOS and the subsequent increase in endothelial cell cGMP levels, we determined whether shear stress af-

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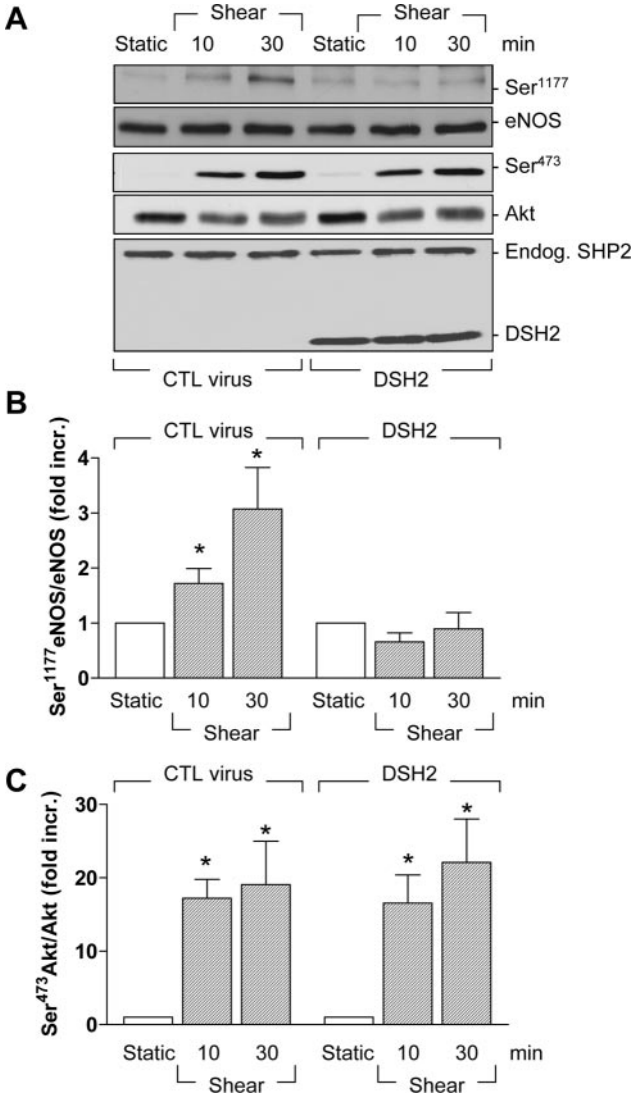

Figure 5. Effect of dominant-negative SHP2 on the shear stress–induced phosphorylation of Akt and eNOS. Primary cultures of porcine aortic endothelial cells were infected with either control virus or with adenovirus encoding DSH2 for 48 hours before stimulation with shear stress (12 dynes/cm^2). A, Representative Western blots showing the effect of the DSH2 on the phosphorylation of eNOS on Ser1177 and Akt on Ser473. The bar graphs (B and C) summarize the data obtained in 4 independent experiments. *$P<0.05$ vs static conditions. incr. indicates increase.

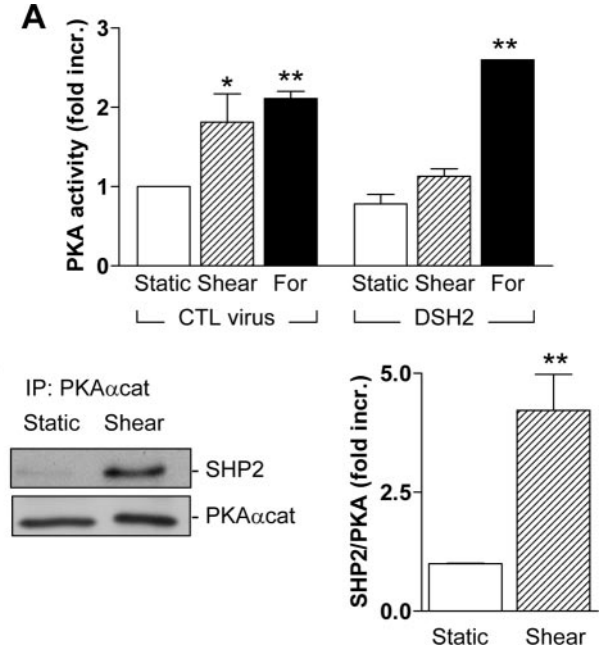

Figure 6. Effect of DSH2 on the shear stress–induced activation of PKA and the effects of shear stress on the association of the 2 proteins. A, Primary cultures of porcine aortic endothelial cells were infected with either control virus or with adenovirus encoding DSH2 for 48 hours before stimulation with shear stress (12 dynes/cm^2), and PKA activity was determined using a commercially available kit. Forskolin (10 μmol/L, 15 minutes) was included as a positive control for PKA activity. B, Coimmunoprecipitation of SHP2 with the catalytic subunit of PKAα (PKAαcat) from cells maintained under static conditions or exposed to shear stress for 30 minutes. Equal pull down of protein was confirmed by reprobing blots for PKAαcat. The bar graphs summarize data obtained in 3 to 4 independent experiments. *$P<0.05$, **$P<0.01$ vs static conditions. incr. indicates increase.

fected PKA activity and whether or not SHP2 was implicated in this process. The stimulation of cultured endothelial cells with fluid shear stress elicited a significant increase in PKA activity, an effect that was attenuated in cells expressing the DSH2 protein (Figure 6A). In parallel experiments, we assessed the association between the catalytic subunit of PKA and SHP2. In cells maintained under static conditions, we failed to detect the association of the 2 proteins, whereas an association was regularly observed in cells exposed to fluid shear stress for 30 minutes (Figure 6B). Although there was no apparent association in cells maintained under static conditions, endothelial cell stimulation with shear stress elicited the time-dependent formation of a PKA protein complex that also included Gab1 and eNOS (Figure 7A). Moreover, in parallel experiments SHP2 was coimmunoprecipitated with eNOS and the association of the 2 proteins increased in response to cell stimulation (Figure 7B).

Flow-Mediated Responses in Carotid Arteries

In isolated phenylephrine-constricted murine carotid arteries, acetylcholine (1 μmol/L) resulted in vasodilatation of approximately 80% (82±11%, n=4), whereas increasing flow resulted in dilatation of up to 60% (Figure 8A). The PKA inhibitor RpAMPS (10 μmol/L) failed to affect the response to acetylcholine (80±4%, n=4, $P=0.87$ versus the solvent-treated group) but markedly blunted flow-induced vasodilatation (Figure 8A).

To determine the role of Gab1 and SHP2 on endothelium-dependent vasodilatation in response to acetylcholine and increased flow, we infected mouse coronary arteries with the corresponding recombinant adenoviruses and monitored flow-induced vasodilatation. Acetylcholine-induced vasodilatation was not significantly different in vessels treated with the green fluorescent protein (GFP), ΔPHGab1, YF-Gab1, or DSH2 viruses; vasodilatation to acetylcholine was 87±5%, 74±4%, 78±4%, and 83±5%, respectively (n=11 to 15, $P=0.36$), indicating that the infection procedure did not compromise endothelial cell function. The efficiency of

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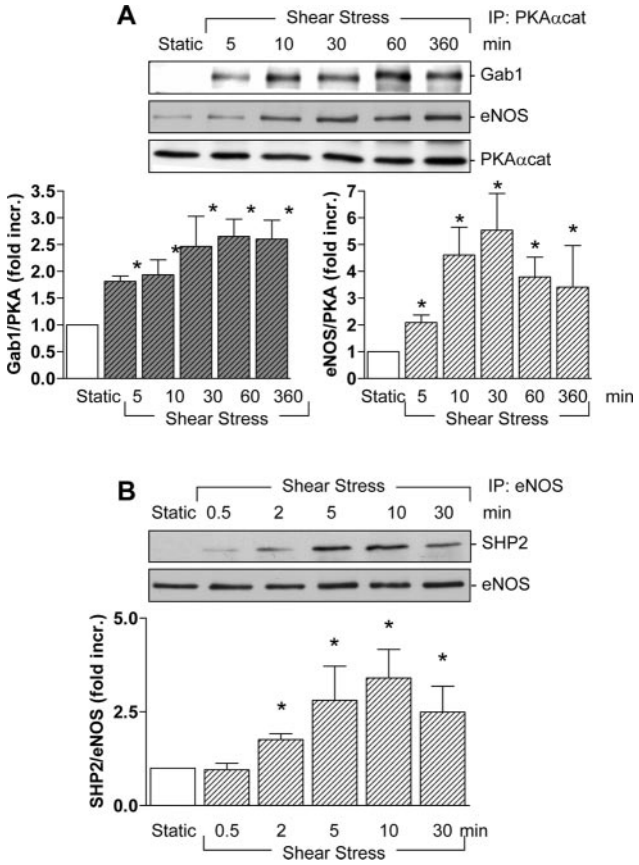

Figure 7. Effect of shear stress on the association of PKA, Gab1, eNOS, and SHP2. A, Coimmunoprecipitation of eNOS and Gab1 with the catalytic subunit of PKAα (PKAαcat) from cells maintained under static conditions or exposed to shear stress for up to 6 hours. B, Coimmunoprecipitation of SHP2 with eNOS from cells maintained under static conditions or exposed to shear stress for up to 30 minutes. The bar graphs summarize data obtained in 3 to 4 independent experiments. *$P<0.05$ vs static conditions. incr. indicates increase.

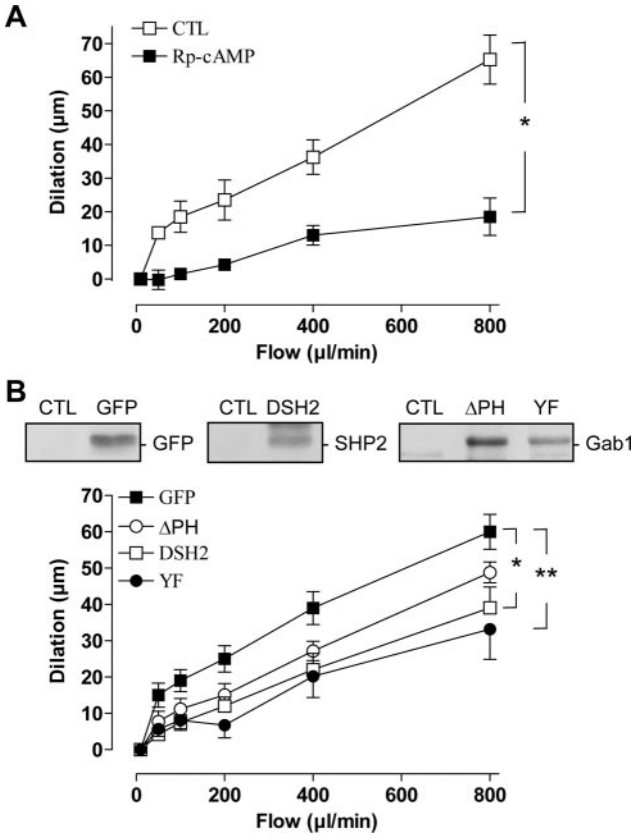

Figure 8. Effect of RpAMPS and overexpression of ΔPHGab1, YF-Gab1, and DSH2 on the flow-mediated vasodilatation of mouse carotid arteries. A, Flow-induced (10 to 800 μL/min) vasodilatation was assessed in freshly isolated mouse carotid arteries in the absence (CTL) and presence of RpAMPS (10 μmol/L). B, Mouse carotid arteries were infected with viruses encoding GFP, ΔPHGab1, YF-Gab1, or DSH2 and maintained in culture for 20 hours before assessing flow-induced vasodilatation. The graphs summarize the results of 4 (A) and 11 to 15 (B) independent experiments. The inset shows overexpression of the respective proteins in carotid arteries. *$P<0.05$, **$P<0.005$ vs responses obtained in the CTL- or GFP-treated groups.

infection was monitored using GFP and confirmed by assessing the expression of individual mutant proteins by Western blot analysis at the end of each experiment (Figure 8B).

Baseline diameter was not significantly different in arteries expressing GFP, ΔPHGab1, YF-Gab1, or DSH2 (501±7, 481±15, 486±8 and 502±9 μm, respectively, n=11 to 15, $P=0.39$). Increasing flow elicited a stepwise vasodilatation that was not significantly different in control and GFP-expressing carotid arteries (compare Figure 8A and 8B), and although flow-induced vasodilatation was slightly attenuated in arteries expressing the ΔPH Gab1 mutant, this effect was not significant ($P=0.16$, n=11). However, flow-induced vasodilatation was significantly attenuated in arteries expressing either DSH2 or the YF-Gab1 mutant (Figure 8B).

Discussion

The results of the present study demonstrate that the shear stress– and the HGF-induced activation of Akt are mediated by signaling pathways differentially dependent on Gab1. Indeed, whereas the pleckstrin homology domain of Gab1 seems to be crucial for the shear stress–induced phosphorylation of Akt, it is completely dispensable for the activation of the kinase by HGF. More importantly, inhibition of the shear stress–induced activation of Akt was without consequence on the phosphorylation and activation of eNOS, which was unchanged because of the activation of PKA. The shear stress–induced phosphorylation and activation of eNOS was, however, causally linked with the Gab1-dependent activation of the tyrosine phosphatase SHP2 inasmuch as a Gab1 mutant (YF-Gab1) to which SHP2 cannot bind, as well as a dominant-negative SHP2 mutant abrogated the shear stress–induced activation of eNOS in cultured as well as in native endothelial cells.

Gab1 is an adapter protein that belongs to the insulin receptor substrate-1 family and is a mammalian homologue of the Drosophila protein DOS (Daughter Of Sevenless), which is involved in multiple signaling events mediated by cytokine and tyrosine kinase receptors, including that of the HGF receptor c-Met.[26] HGF has previously been reported to enhance the phosphorylation of eNOS on Ser1177 and increase endothelial NO production.[10] However, although interfering with the function of Gab1 is reported to attenuate

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the activation of the PI3-K and Akt in several different cell types in response to growth factor stimulation,[27] we observed no effect of either ΔPHGab1 or the YF-Gab1 mutant on the activation of Akt or eNOS in HGF-stimulated endothelial cells. The latter observations cannot rule out a role for Gab1 in the HGF-induced activation of PI3-K and Akt in endothelial cells as the binding of Gab1 to the c-Met receptor is thought to be dependent on its Met-binding sequence,[28] which remained intact in both of the Gab1 mutants assessed in the present study.

There does, however, appear to be a role for Gab1 in regulating the shear stress–induced activation of Akt as the ΔPHGab1 mutant abrogated the shear stress–induced phosphorylation of Akt. This indicates that the translocation of Gab1 to the plasma membrane plays an important role in the regulation of Akt by fluid shear stress. Indeed, a second Gab1 mutant (YF-Gab1), which should be able to translocate normally within endothelial cells but is unable to bind SHP2, did not prevent the shear stress–induced phosphorylation of Akt.

Fluid shear stress elicits the phosphorylation of eNOS on Ser1177 and although we and others initially implicated Akt in this process,[6] the results of the present investigation revealed that a kinase other than Akt must be responsible for the shear stress–induced phosphorylation and activation of eNOS. This conclusion is based on the observations that ΔPH Gab1 failed to affect the shear stress–induced phosphorylation of eNOS, although the activation of Akt was abrogated, and that the YF-Gab1 mutant abolished the phosphorylation of eNOS while leaving the shear stress–induced activation of Akt intact. During the preparation of this manuscript, Jin et al[29] reported that Gab1 regulates the phosphorylation and activation of eNOS via Akt as the downregulation of Gab1 (using a siRNA approach) and a Gab1 mutant that was unable to bind PI3-K attenuated both processes. Although the downregulation of Gab1 also prevented the shear stress–induced phosphorylation of Akt and eNOS in our experiments, studies aimed at determining the consequences of the specific mutation of Gab1 indicate that Akt is not responsible for the phosphorylation of eNOS. Despite the apparent contradiction, the report by Jin et al[29] is not in direct conflict with the results shown here. Indeed, preventing the shear stress–induced activation of PI3-K would be expected to affect the activation of both Akt and PKA,[2] and the global downregulation of Gab1 would be expected to affect both the activation of Akt, which we propose requires the translocation of Gab1 to the plasma membrane, as well as the association of SHP2 with Gab1, which is dependent on the phosphorylation of Tyr627 and which does affect the shear stress–induced phosphorylation of eNOS.

Several events in the signaling cascade activated by hemodynamic stimuli in endothelial cells are associated with tyrosine phosphorylation and/or dephosphorylation (for review, see Shyy and Chien[30]). The fact that both the YF-Gab1 mutant, which cannot bind SHP2,[20] and the dominant-negative SHP2 mutant prevented the shear stress–induced phosphorylation and activation of eNOS, indicate that SHP2 is also an integral part of this signal transduction process. Fluid shear stress is known to attenuate the tumor necrosis factor-α–induced activation of SHP2 in endothelial cells,[31] but, to the best of our knowledge, this is the first report demonstrating the involvement of SHP2 in regulating the shear stress–induced activation of eNOS. Little is known about substrates for SHP2 in endothelial cells, but eNOS itself is reported to be tyrosine phosphorylated,[22] and SHP2 has been implicated in the regulation of neuronal NOS activity.[32] As we were able to detect SHP2 as part of the eNOS signalosome in shear stress–stimulated cells, it is likely that either eNOS or an eNOS-associated protein is a SHP2 substrate.

Given that our findings indicate that the phosphorylation and activation of eNOS in response to shear stress is largely independent of Akt, we assessed the role of PKA in response to shear stress. We observed that a PKA inhibitor abrogated the phosphorylation of eNOS on Ser1177, prevented the shear stress–induced accumulation of cGMP, and significantly attenuated the flow-induced vasodilatation of mouse carotid arteries. These data confirm and extend previously published reports on the importance of PKA in mediating eNOS activation in response to fluid shear stress,[11] although it is unclear how the activity of PKA is stimulated under the conditions studied. Our data suggest a link between SHP2 and the activation of PKA inasmuch as shear stress stimulated the association of SHP2 with the catalytic subunit of PKA and the shear stress–induced activation of PKA was attenuated in endothelial cells expressing the DSH2 mutant. Because shear stress increases PKA activity without increasing endothelial cAMP levels,[33] and PKA, SHP2, Gab1, and eNOS coexist as a signalosome complex in shear stress–stimulated endothelial cells, it is tempting to suggest that SHP2 can directly regulate PKA activity.

In conclusion, our data clearly indicate that the adaptor protein Gab1 and the tyrosine phosphatase SHP2 play crucial roles in the activation of eNOS by fluid shear stress in cultured and native endothelial cells. The shear stress–induced phosphorylation of Gab1 and the association with SHP2 are essential for the activation of PKA, which is ultimately responsible for the increase in endothelial NO production.

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