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

Madhulika Dixit, Annemarieke E. Loot, Aniszuddin Mohamed, Beate Fisslthaler, Chantal M. Boulanger, Bogdan Ceacareanu, Aviv Hassid, Rudi Busse, Ingrid Fleming

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

Although the endothelial NO synthase (eNOS) was initially described as a Ca2+-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 Ca2+-elevating agonists as well as to hemodynamic stimuli, such as cyclic stretch and fluid shear stress (for reviews, see Fleming and Busse and Boo and Jo). 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. The dephosphorylation of Thr495 facilitates the Ca2+-dependent association of calmodulin with eNOS, whereas the phosphorylation of eNOS on Ser1177 increases NO output in an apparently Ca2+-independent manner. As the maintained production of endothelium-derived NO in response to fluid shear stress is a Ca2+-dependent process, 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 Ca2+/calmodulin-dependent kinase II in bradykinin-stimulated endothelial cells but to Akt in cells stimulated with vascular endothelial growth factor or hepatocyte growth factor (HGF) and to Akt and, probably more importantly, to protein kinase A (PKA) 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). We and others have...
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 Newman15 and Ilan and Madri16). Indeed, Gab-1 translocates from the cytoplasm to endothelial cell junctions in response to flow17 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–phospho-tyrosine 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/cm2 in a cone-plate viscometer as described22 or to HGF (50 ng/mL). In some experiments, the intracellular concentration of cGMP was measured in the cell supernatants or immunoprecipitates were subjected to SDS-PAGE.8 Proteins were detected using respective antibodies and visualized by enhanced chemiluminescence using a commercially available kit (Amersham, Freiburg, Germany) as described22 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.8 Proteins were detected using respective antibodies and visualized by enhanced chemiluminescence using a commercially available kit (Amersham, Freiburg, 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 (3×106 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 (3×106 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 CO2 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-recorded 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/cm2) 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).
Ser473 was low. Both fluid shear stress (12 dynes/cm²) and HGF, another stimulus for PI3-K and Akt activation, elicit the phosphorylation of Akt in endothelial cells stimulated by either fluid shear or exposed to shear stress. The blots shown are a representative of 3 independent experiments. incr. indicates increase.

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/cm²) 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, 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/cm²) 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 inability 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, may prevent the shear stress–induced activation of eNOS.
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-
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.

**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/cm², 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 vs static conditions. 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/cm²). 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.

**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-
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, 15 minutes) was included as a positive control for PKA activity. B, Coimmunoprecipitation of SHP2 with the catalytic subunit of PKA (PKAcat) 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 PKAcat. The bar graphs summarize data obtained in 3 to 4 independent experiments. *P<0.05, **P<0.01 vs static conditions. incr. indicates increase.

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/cm²). 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/cm²), 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 PKA (PKAcat) 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 PKAcat. The bar graphs summarize data obtained in 3 to 4 independent experiments. *P<0.05, **P<0.01 vs static conditions. incr. indicates increase.
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, H9004PHGab1, YF-Gab1, or DSH2 (501 ± 17, 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 H9004PH 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 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. HGF has previously been reported to enhance the phosphorylation of eNOS on Ser1177 and increase endothelial NO production. However, although interfering with the function of Gab1 is reported to attenuate 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.
the activation of the PI3-K and Akt in several different cell types in response to growth factor stimulation, 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, 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, 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 al29 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 SHP2, did not prevent the shear stress–induced phosphorylation of Akt.

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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Preparation and expression of adenoviral vectors

Recombinant adenoviruses expressing enhanced green fluorescence protein (GFP) were generated by cloning the cDNA obtained from BD Biosciences (San Jose, USA) as an EcoRI-BamHI fragment in pShuttle. Plasmid pcDNA 3HAGab1, was kindly provided by Dr. Keigo Nishida (Osaka, Japan). Recombinant adenoviruses expressing WT-Gab1 and Y627F-Gab1 (YF-Gab1) were generated as previously described. To generate recombinant adenovirus lacking the N-terminal pleckstrin homology domain of Gab1 (ΔPH-Gab1), an inverse PCR strategy was employed using pShuttleGab1 as template following a published protocol. The following primers were used: forward primer: 5’phos-GCACACAGAAGAAGATCCTGTGAAGCCAC-3’, reverse primer 5’phos-GCACACAGAAGAAGATCCTGTGAAGCCAC-3’. The desired deletion of the PH domain was confirmed via sequencing prior to generation of recombinant adenoviruses. The adenoviruses were purified using Adeno-X™ Virus Purification Kit from BD Biosciences (Heidelberg, Germany) and the plaque forming units were determined by plaque assay in HEK 293 cells. Recombinant adenoviruses expressing the SHP2 mutant, DSH2 were generated as described. For efficient expression of the recombinant proteins, confluent primary cultures of porcine aortic endothelial cells were infected with the respective adenovirus (3x10⁶ pfu/mL). Four hours after infection in serum-free medium, cells were washed extensively in phosphate-buffered saline and fresh medium containing 4% fetal calf serum (FCS) was added and cells were further incubated for an additional 44 hours.

Adenoviral infection of mouse carotid arteries

Male C57BL/6 mice, six to nine weeks of age (Charles River, Sulzfeld, Germany) were anaesthetized with isoflurane. Via a laparotomy, the intestines and the diaphragm were removed, and a needle was placed in the left ventricle into which saline was infused to flush the blood out of the circulation. The thorax was opened, and the carotid arteries and the aortic root were nominally freed of connective tissue but maintained in situ. Around each carotid artery, two prolene 6-0 ligatures were placed, but not tightened. A catheter filled with MCDB 131 culture medium containing 2 % FCS was advanced via the aorta into the carotid and secured with the proximal ligature. With a Hamilton syringe a small air bubble followed by 20 µl virus solution (3x10⁶ pfu) were brought in the catheter. By infusing more MCDB medium the bubble was advanced beyond the bifurcation, and the distal ligature was tightened just below the bifurcation. To slightly pressurize the vessel, 5 µl more medium was infused. The proximal suture was then slipped off the catheter and tightened around the carotid. The procedure was repeated...
with the second carotid artery. During the entire procedure the vessels were kept moist with MCDB medium. The filled carotid arteries were excised, and placed in culture medium in a standard CO₂ incubator (37°C) for four hours. Thereafter, the ligatures were removed, the vessels washed extensively (at least 5 times) to remove the virus and then maintained overnight in culture medium. The distal ends were cut diagonally to allow proper orientation in the perfusion setup. At the end of each experiment to assess flow-induced vasodilatation the segments were incubated with lysis buffer and subjected to Western blotting to determine the effectiveness of the adenoviral infection. The infection procedure used resulted only in the infection of the endothelial cell layer, as demonstrated in Figure S-1.

**Figure S-1.** En face confocal images showing the expression of GFP and the HA-tagged Gab1 mutants in the endothelial cells of a mouse carotid artery. Mouse carotids were infected with an adenoviruses encoding for GFP, HA-tagged Gab1-ΔPH, or HA-tagged Gab1-YF. The vessels were cut open longitudinally, partially endothelium-denuded, fixed in formalin and incubated with antibodies directed against PECAM-1 or the HA-tag. The localization of the transgenes was assessed by confocal laser microscopy. The dotted line represents the boundary between the endothelium-intact and endothelium-denuded areas.
Assessment of NO production

**Electron spin resonance (ESR) spectroscopy:** NO production was detected using colloidal iron (II) diethylthiocarbamate [Fe(DETC)₂; 100 µmol/L] as a spin trap⁴ in cells incubated in HEPES buffer (mmol/L: NaCl 98.93, KCl 4.69, MgSO₄·7H₂O 1.2, CaCl₂ 2.49, NaHCO₃ 25.0, K₂HPO₄·3H₂O 0.61, glucose 11.1 and HEPES 21.85; pH 7.4) at 37°C for 30 minutes. Thereafter, the cells were scraped, collected in an insulin syringe and frozen in liquid nitrogen. The formation of paramagnetic mono-nitrosyl-iron complexes [NO-Fe(DETC)₂], was determined at 77 K in a liquid nitrogen cooled dewar using a EPR EMX spectrometer (X-band, Bruker; Karlsruhe, Germany). The instrument settings were as follows: microwave frequency, 9.487 GHz; power, 20.07 mW; receiver gain, 1x10⁵; modulation amplitude, 5.000G; modulation frequency, 100 KHz; conversion time, 81.92ms; time constant, 327.680ms; sweep time, 41.94s; sweep width, 100G; resolution, 512 points and 3 scans. After correction of baseline by subtraction of the L-NAME (300 µmol/L, 45 minutes) insensitive signal, the amplitude of the NO-Fe(DETC)₂ EPR triplet signal was taken as a measure for NO release.

**NO electrode:** Porcine aortic endothelial cells were grown on quartz coverslips and studied either uninfected or approximately 40 hours after infection with ∆PHGab1 adenoviruses (3x10⁶ pfu/mL). Coverslips were placed perpendicularly in a cuvette containing HEPES-Tyrode solution (mmol/L: NaCl 132, KCl 4.7, CaCl₂ 1.0, MgCl₂ 1.0, NaH₂PO₄ 0.36, HEPES 10, Glucose 5, pH 7.4 37°C) and a magnetic stirrer to facilitate rapid distribution of compounds. After calibration as described,⁵ the NO-sensitive microelectrode (ISONOP30, World Precision Instruments, Stevenage, UK) was inserted into the cuvette and placed close to the endothelial surface. In some experiments, calibration of the electrode was performed before and after the experimental protocol to ensure that the sensitivity remained unchanged, a lack of response to sodium nitrite (up to 10 µmol/L) and noradrenalin (0.5–1 µmol/L) in the absence of endothelial cells, was taken as evidence for an intact coating of the electrode.⁶

**Effect of wild-type Gab1 and the ∆PHGab1 mutant on the activation of eNOS**

Treatment of porcine endothelial cells with recombinant adenoviral vectors for wild-type Gab1 and ∆PHGab1 for 48 hours resulted in significant overexpression of the two proteins as compared to cells treated with empty vector control (Figure S-2A). NO spin trapping with Fe(DETC)₂ revealed the characteristic anisotropic triplet signal for NO, that was sensitive to preincubation with the NOS inhibitor Nω-nitro-L-arginine methyl ester (L-NAME, 300 µmol/L; Figure S-2A). Overexpression of wild-type Gab1 increased basal NO production while the ∆PHGab1 mutant attenuated NO generation (Figure S-2A&B). HGF (50 ng/mL) stimulated the production of NO from cultured porcine endothelial cells. Incubation of the cells with the control adenovirus slightly attenuated the HGF-induced
increase in NO production and no difference in the response to HGF was detected in cells treated with the control virus and in cells expressing the \( \Delta PH\text{Gab}1 \) mutant (Figure S-2C).

**Figure S-2. Effect of the overexpression of wild-type Gab1 and the \( \Delta PH\text{Gab}1 \) mutant on NO production.** Porcine aortic endothelial cells were infected with either control viruses or viruses encoding either wild-type (WT) Gab1 or the \( \Delta PH\text{Gab}1 \) mutant 48 hours prior to incubation with the NO spin trap Fe(DET)\( \text{C}_2 \). NO production was monitored by ESR spectroscopy using cells maintained under basal conditions for 30 minutes. (A) Characteristic anisotropic signals for the NO-Fe(DET)\( \text{C}_2 \) complex. The insert shows the overexpression of WT Gab1 and \( \Delta PH\text{Gab}1 \) mutant. (B) Bar graph summarizing the data from 4 independent experiments; **P<0.01. (C) The increase in NO production (\( \Delta [\text{NO}] \)) elicited by HGF (50 ng/mL) in uninfected cells and in cells infected with the control (CTL) or \( \Delta PH\text{Gab}1 \) viruses. The response to ionomycin was included as a positive control. The bar graph summarizes data obtained in 5 separate experiments.
Effect of PKA inhibitors on the phosphorylation and activation of eNOS and Akt

As PKA can be activated by fluid shear stress and can also phosphorylate eNOS on Ser\textsuperscript{1177}, we determined the effects of a PKA inhibitor on the shear stress-induced phosphorylation of eNOS on Ser\textsuperscript{1177}. The PKA inhibitors RpAMPS (10 µmol/L) and H89 (10 µmol/L) completely prevented the shear stress-induced increase in the phosphorylation of eNOS Ser\textsuperscript{1177}, without influencing that of Akt (Figure S-3).

Figure S-3. Effect of PKA inhibitors on the shear stress-induced phosphorylation of eNOS and Akt in confluent cultures of porcine aortic endothelial cells. Bar graphs summarizing (3 independent experiments) the effect of RpAMPS (10µmol/L) and H89 (10µmol/L) on the shear stress-induced phosphorylation of (A) eNOS on Ser\textsuperscript{1177} and (B) Akt on Ser\textsuperscript{473}; *P<0.05, **P<0.01 versus static conditions.

Knock-down of endogenous Gab1 using small interfering RNA (siRNA)

A commercially available kit (SMART pool) was used according to the manufacturers instructions (Upstate). Confluent primary cultures of human umbilical vein endothelial cells were starved of serum for 4 hours prior to transfection. Cells were transfected with Gab1 siRNA (50 µmol/L) with the aid of transfection reagent SilMPORTER (Upstate). After 72 hours, cells were either maintained under static conditions or exposed to fluid shear stress (12 dynes/cm\textsuperscript{2}) for the times indicated.

Specific knock-down of endogenous Gab1 using this method was confirmed by performing Western blot analysis of the cell lysates (Figure S-4). Following the downregulation of Gab1, shear stress failed to elicit either the phosphorylation of Akt or of eNOS (Figure S-4).
Figure S-4. Consequences of the down regulation of Gab1 on the shear stress-induced phosphorylation of Akt and eNOS.

Confluent cultures of human umbilical vein endothelial cells were treated with control oligos or Gab1 siRNA for 72 hours prior to stimulation with shear stress (12 dynes/cm²) for 10 or 30 minutes. (A) Representative Western blots showing the effect of the Gab1 siRNA on the phosphorylation of eNOS on Ser^{1177} and Akt on Ser^{473}. The bar graphs (B and C) summarize data obtained in 4 independent experiments; **P<0.01 and ***P<0.001 versus static conditions.
References


