

Role of PECAM-1 in the shear-stress-induced activation of Akt and the endothelial nitric oxide synthase (eNOS) in endothelial cells

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Accepted 13 June 2005

Journal of Cell Science 118, 4103–4111 Published by The Company of Biologists 2005

doi:10.1242/jcs.02541

Summary

The application of fluid shear stress to endothelial cells elicits the formation of nitric oxide (NO) and phosphorylation of the endothelial NO synthase (eNOS). Shear stress also elicits the enhanced tyrosine phosphorylation of endothelial proteins, especially of those situated in the vicinity of cell-cell contacts. Since a major constituent of these endothelial cell-cell contacts is the platelet endothelial cell adhesion molecule-1 (PECAM-1) we assessed the role of PECAM-1 in the activation of eNOS.

In human endothelial cells, shear stress induced the tyrosine phosphorylation of PECAM-1 and enhanced the association of PECAM-1 with eNOS. Endothelial cell stimulation with shear stress elicited the phosphorylation of Akt and eNOS as well as of the AMP-activated protein kinase (AMPK). While the shear-stress-induced tyrosine phosphorylation of PECAM-1 as well as the serine phosphorylation of Akt and eNOS were abolished by the pre-treatment of cells with the tyrosine kinase inhibitor

PP1 the phosphorylation of AMPK was unaffected. Down-regulation of PECAM-1 using a siRNA approach attenuated the shear-stress-induced phosphorylation of Akt and eNOS, as well as the shear-stress-induced accumulation of cyclic GMP levels while the shear-stress-induced phosphorylation of AMPK remained intact. A comparable attenuation of Akt and eNOS (but not AMPK) phosphorylation and NO production was also observed in endothelial cells generated from PECAM-1-deficient mice.

These data indicate that the shear-stress-induced activation of Akt and eNOS in endothelial cells is modulated by the tyrosine phosphorylation of PECAM-1 whereas the shear-stress-induced phosphorylation of AMPK is controlled by an alternative signaling pathway.

Key words: Mechanotransduction, siRNA, AMP-activated protein kinase (AMPK)

Introduction

Endothelial cells situated at the interface between blood and the vessel wall, play a crucial role in controlling vascular tone and homeostasis, particularly in determining the expression of pro- and anti-atherosclerotic genes. Many of these effects are mediated by changes in the generation and release of the vasodilator nitric oxide (NO) in response to hemodynamic stimuli exerted on the luminal surface of endothelial cells by the streaming blood (wall shear stress) and the cyclic strain of the vascular wall, which results from the pulsatile changes in blood pressure (for review, see Busse and Fleming, 2003).

NO is generated by the endothelial NO synthase (eNOS), a Ca²⁺/calmodulin-dependent enzyme, the activity of which can also be regulated by phosphorylation on specific serine and threonine residues (Fleming and Busse, 2003; Boo and Jo, 2003). Following the application of shear stress to endothelial cells NO production is enhanced twofold to fourfold over basal values and is maintained as long as the stimulus is applied. The first step in the process that translates a physical stimulus into an increase in NO production was initially thought to be the activation of a specific mechanoreceptor (such as a mechanosensitive ion channel or a component of the glycocalyx) but current thinking tends towards classifying the entire

endothelial cell cytoskeleton as a mechanoreceptor (for recent reviews see Davies et al., 2003; Ingber, 2003). Thus, the application of a mechanical stimulus can be transmitted through the entire cell by the cytoskeleton to activate signal transduction cascades in 'signaling hot spots'. Although shear stress and cyclic stretch can activate signaling cascades within caveolae (Rizzo et al., 1999) and focal adhesion contacts (Vuori, 1998), the lateral zone of cell-cell adhesion is thought to be the major signal transduction site for fluid shear stress (Kano et al., 2000; Fujiwara et al., 2001; Davies et al., 2003).

The platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) is concentrated at cell-cell contacts and undergoes homophilic binding between adjacent endothelial cells. Initially it was attributed a function in the regulation of leukocyte transmigration, cell migration, cell adhesion and angiogenesis (DeLisser et al., 1997) but more recently PECAM-1 was found to contain two intracytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIMs), centered around Tyr⁶⁶³ and Tyr⁶⁸⁶, which has led to a reconsideration of its role in cell signaling (Newman, 1999). Although the fact that PECAM possesses ITIMs would tend to suggest that any potential role in signaling is modulatory rather than activatory, PECAM-1 is known to become rapidly

tyrosine-phosphorylated following the application of fluid shear stress to endothelial cells grown under static conditions (Harada et al., 1995; Osawa et al., 1997). As this response cannot be mimicked by Ca^{2+} -elevating agonists or growth factors it was suggested that PECAM-1 may represent a mechanoreceptor or part of such a complex on the endothelial cell surface (Osawa et al., 1997; Osawa et al., 2002). Thus, PECAM-1 appears to be exquisitely sensitive to fluid shear stress and is situated in a subcellular compartment that is rich in many of the signaling molecules known to be activated following the application of fluid shear stress to endothelial cells, including eNOS (Govers et al., 2002). The aim of the present investigation was to determine whether PECAM-1 is involved in the shear-stress-induced activation of eNOS. To this end we assessed the shear-stress-induced phosphorylation of PECAM, Akt and eNOS as well as eNOS activity in human endothelial cells under basal conditions as well as following the downregulation of PECAM-1 and compared the responses obtained with those of wild-type and PECAM-1-deficient murine endothelial cells.

Materials and Methods

Materials

Antibodies for western blotting and immunohistochemistry directed against Ser¹¹⁷⁷eNOS, Ser⁴⁷³ Akt, Thr¹⁷² AMPK and Tyr⁴¹⁶ Src were from Cell Signaling (New England Biolabs, Frankfurt, Germany), the antibodies against β -catenin and PECAM-1 were from Santa Cruz (Heidelberg, Germany), eNOS from Becton Dickinson (Heidelberg, Germany), c-Src and phospho-tyrosine (clone AG10) from Upstate (Biomol, Hamburg, Germany) and β -actin from Sigma (Munich, Germany). Vascular endothelial growth factor (VEGF) was from Cell Concepts GmbH (Umkirch, Germany), PP1 and PP3 were from Calbiochem (Bad Soden, Germany) and all other chemicals were from Sigma (Munich, Germany).

Cell culture

Human endothelial cells

Human umbilical vein endothelial cells were isolated and cultured as described (Busse and Lamontagne, 1991). Due to the loss of several signaling pathways with time in culture, endothelial cells were used after one passage.

Murine endothelial cells

Murine endothelial cells were isolated and purified by slight modifications of procedures described (Marelli-Berg et al., 2000; Kataoka et al., 2003). Six-week-old mice; C57 BL/6 (Charles Rivers) or PECAM-1-deficient (PECAM-1^{-/-}) mice (Duncan et al., 1999), that had been backcrossed onto a C57 BL/6 background and kindly provided by Peter J. Newman (Milwaukee, WI), were sacrificed by an overdose of isoflurane (four mice per preparation); the lungs were removed, minced with a scalpel, washed in Hank's buffer and incubated with dispase (20 ml, 5 U ml⁻¹, 1 hour). Thereafter, the homogenate was filtered through a cell strainer (40 μm ; Becton Dickinson, Heidelberg, Germany), collected by centrifugation (290 g, 5 minutes) and washed with phosphate-buffered saline containing 0.1% bovine serum albumin (PBS/BSA). The resulting cell suspension was incubated with anti-mouse VE-cadherin antibody (Pharmingen, Becton Dickinson, Heidelberg, Germany)-coated magnetic beads (DynaL Biotech, Hamburg, Germany) for 30 minutes. The magnetic beads were washed four times with PBS/BSA, resuspended in D-MEM/F12 (Invitrogen, Karlsruhe, Germany) supplemented with 20% fetal calf serum (Biochrom, Berlin,

Germany), endothelial cell growth factor (Promocell, Heidelberg, Germany), penicillin (50 U ml⁻¹) and streptomycin (50 μg ml⁻¹) and seeded on gelatin-coated culture dishes. For the first two passages the cells were re-purified with VE-cadherin antibody-coated magnetic beads. This purification protocol resulted in cell cultures that tested more than 95% Dil-Ac-LDL positive. In the present study cells in the third passage were used.

Agonist stimulation and shear stress

Confluent endothelial cells were stimulated following serum starvation for 4 hours; human endothelial cells were washed twice with and incubated in M-199 medium containing 0.1% BSA while the murine endothelial cells were incubated in D-MEM/F12 containing 0.1% BSA. Thereafter cells were stimulated with solvent (culture medium or 0.01% DMSO), VEGF (100 ng ml⁻¹, up to 30 minutes), histamine (1 μmol l⁻¹, up to 30 minutes), bradykinin (10 nmol l⁻¹, 5 minutes) or ionomycin (0.1 μmol l⁻¹, up to 30 minutes). Alternatively, cells were either maintained under static conditions or subjected to shear stress of 12 dynes cm⁻² in a cone-plate viscosimeter as described (Fleming et al., 1998) for up to 2 hours in the absence and presence of PP1 (30 μmol l⁻¹) and wortmannin (40 nmol l⁻¹). In some experiments the intracellular concentration of cyclic GMP was measured in presence of a phosphodiesterase inhibitor (isobutyl-methylxanthine; 50 μmol l⁻¹) using a specific radioimmunoassay (Amersham, Freiburg, Germany) according to the manufacturer's instructions.

Immunoprecipitation and SDS-PAGE

Following stimulation, cells were solubilised in lysis buffer (Tris/HCl pH 7.5; 50 mmol l⁻¹; NaCl, 150 mmol l⁻¹; EGTA, 2 mmol l⁻¹; EDTA 2 mmol l⁻¹; Triton X-100, 1% (v/v); NaF, 25 mmol l⁻¹; Na₄P₂O₇, 10 mmol l⁻¹; 2 μg ml⁻¹ each of leupeptin, pepstatin A, antipain, aprotinin, chymostatin and trypsin inhibitor, and PMSF, 40 μg ml⁻¹), left on ice for 10 minutes and centrifuged at 10,000 g for 10 minutes. In some experiments PECAM-1 was immunoprecipitated using a specific antibody and the antigen/antibody complexes were recovered using protein A/G sepharose (50 μl , 2 hours). After centrifugation (10,000 g, 10 minutes) the pellets were washed four times with Tris buffer (Tris/HCl pH 7.5, 50 mmol l⁻¹; NaCl, 150 mmol l⁻¹; EDTA, 2 mmol l⁻¹; EGTA, 2 mmol l⁻¹) and twice in the same buffer containing Tween 20 (0.1% v/v). Proteins in the cell supernatants or immunoprecipitates were heated with SDS-PAGE sample buffer, separated by SDS-PAGE and subjected to western blotting as described (Fleming et al., 2001). Proteins were detected using their respective antibodies, and visualized by enhanced chemiluminescence using a commercially available kit (Amersham, Germany). To assess the phosphorylation of proteins, either equal amounts of protein from each sample were loaded twice and one membrane incubated with a phospho-specific antibody and the other with an antibody recognizing total protein, or blots were reprobbed with the appropriate antibody.

Small interfering RNA (siRNA)

The human cDNA sequence of PECAM-1 was scanned for putative target sequences for siRNA by Eurogentec (Searing, Belgium) and PECAM-1 specific RNA oligonucleotides with the sequences GGCC-CCAAUACACUUCACAdTdT and AACCACUGCAGAGUACCAG-dTdT (corresponding to position 687-705 and 586-585 in the PECAM-1 cDNA sequence accession number: BC051822) as well as oligonucleotides with the random sequence GCCAAACGAUCCC-AUACCUdTdT were synthesized (Eurogentec, Searing, Belgium). The siRNA was generated by annealing of the sense and antisense RNA oligonucleotides by heating to 95°C and slowly cooling to room temperature in 50 mmol l⁻¹ Tris/HCl; 100 mmol l⁻¹ NaCl pH 7.5. The duplexes (120 pmol per 3.5 cm culture dish) were introduced into subconfluent (~75-80%) cultured human umbilical vein endothelial

cells using the liposomal reagent Genetrans II (MoBiTec, Göttingen, Germany) according to the manufacturer's instructions. After 48 hours the cells were transferred to medium containing 2% FCS and four hours thereafter exposed to shear stress or bradykinin for the times indicated in the result section.

Immunohistochemistry

Endothelial cells were grown on glass slides, treated with transfection reagent, scrambled oligonucleotides or siRNA oligonucleotides as described above and cultured for 48 hours. The cells were fixed with formaldehyde (2% in PBS), permeabilized with Triton X-100 (0.05%) and incubated in PBS containing glycine (2%) for 10 minutes. Cells were co-incubated with the nuclear marker TOPRO-3 (Molecular Probes, Leiden, The Netherlands) and a specific PECAM-1 monoclonal antibody (DakoCytomation; Glostrup, Denmark) followed by an Alexa dye-coupled secondary antibody (Molecular Probes). Following incubation, the preparations were mounted and viewed using a confocal microscope.

Statistics

Data are expressed as the mean±s.e.m. Statistical analyses were performed by one-way ANOVA followed by Bonferroni's multiple comparison test. Values of *P*<0.05 were considered statistically significant.

Results

Effect of shear stress on tyrosine phosphorylation of PECAM-1 and c-Src

In confluent cultures of human endothelial cells, the application of fluid shear stress (12 dynes cm⁻²) elicited the marked tyrosine phosphorylation of PECAM-1. The tyrosine phosphorylation, assessed by immunoprecipitating PECAM-1 and determining its tyrosine phosphorylation, was detectable in cells exposed to fluid shear stress for two minutes and was maintained as long as the stimulus was applied, i.e. up to 120 minutes (Fig. 1A). The Ca²⁺-elevating agonists, bradykinin (data not shown), VEGF (100 ng ml⁻¹), histamine (1 μmol l⁻¹) and ionomycin (0.1 μmol l⁻¹) elicited at best a weak phosphorylation of PECAM-1 which was markedly less than the phosphorylation elicited by fluid shear stress (Fig. 1B).

As Src- and Csk-related protein tyrosine kinases can phosphorylate PECAM-1 (Cao et al., 1998), and c-Src can be co-precipitated with PECAM-1 from shear stress-stimulated endothelial cells (Osawa et al., 1997), we determined whether or not the time course of Src activation by fluid shear stress coincides with that of the tyrosine phosphorylation of PECAM-1.

In cultured human endothelial cells, exposure to fluid shear stress resulted in a significant increase in the phosphorylation of Src on Tyr⁴¹⁶ (Fig. 2A), which is generally consistent with the activation of the kinase (Helmke et al., 1998). In the endothelial cells used, the shear-stress-induced phosphorylation of Src was maintained as long as the stimulus was applied. This time course of Src activation is consistent with observations made by other groups (Jalali et al., 1998) and correlates well with the time course of PECAM-1 tyrosine phosphorylation. The Src-kinase family inhibitor, PP1 (30 μmol l⁻¹) completely prevented the shear-stress-induced tyrosine phosphorylation of PECAM-1 (Fig. 2B).

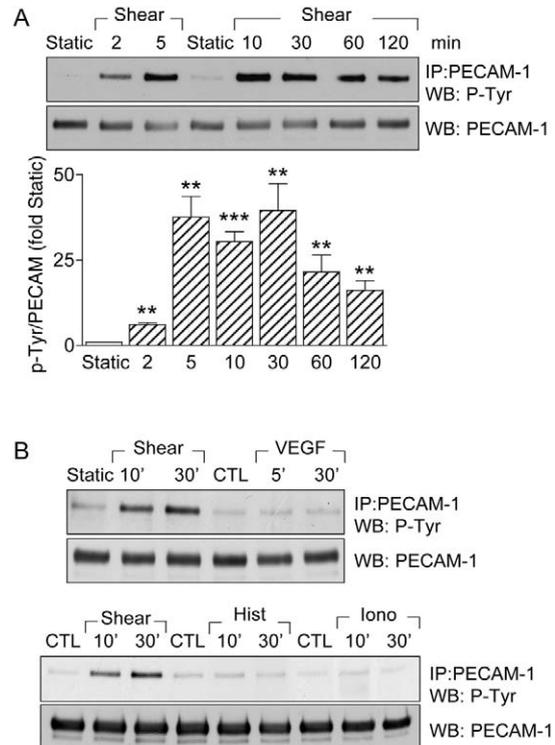


Fig. 1. Effects of shear stress and receptor-dependent agonists on the tyrosine phosphorylation of PECAM-1. Primary cultures of human umbilical vein endothelial cells were either maintained under static conditions, exposed to fluid shear stress (Shear; 12 dynes cm⁻², 2-120 minutes) or stimulated with either solvent (CTL), VEGF (100 ng ml⁻¹, 5-30 minutes), histamine (Hist; 1 μmol l⁻¹, 10-30 minutes), or ionomycin (Iono; 0.1 μmol l⁻¹, 10-30 minutes). PECAM-1 was immunoprecipitated and the tyrosine phosphorylation was assessed by western blotting. To verify equal amounts of immunoprecipitated protein the blot was reprobed with an antibody raised against PECAM-1. The western blots shown are representative of data obtained in three independent experiments and the bar graph summarizes data obtained in three independent experiments; ***P*<0.01, ****P*<0.001 versus static conditions.

Effect of shear stress on association of eNOS with PECAM-1 and eNOS phosphorylation

In cells maintained under static conditions we detected only a weak association between eNOS and PECAM-1. However, this protein-protein interaction increased following exposure to fluid shear stress for up to 60 minutes (Fig. 3A).

We next determined whether or not PP1 affected the shear-stress-induced phosphorylation of Akt and eNOS. In cells under static conditions low levels of Akt (Ser⁴⁷³) and eNOS (Ser¹¹⁷⁷) phosphorylation were detected and the application of shear stress (12 dynes cm⁻², 30 minutes) was associated with the phosphorylation of Akt as well as that of eNOS (Fig. 3B). The Src-family tyrosine kinase inhibitor PP1 (30 μmol l⁻¹) markedly attenuated the phosphorylation of Akt and eNOS while the inactive compound PP3 failed to affect the response to shear stress.

In the same experiments we assessed the effect of shear stress and PP1 on the phosphorylation of the AMPK, which has also been reported to phosphorylate eNOS (Chen et al.,

1999) but is generally activated in response to glucose starvation (Ruderman et al., 2003) and hypoxia (Nagata et al., 2003; Zou et al., 2003). We observed a shear stress-dependent phosphorylation of the AMPK on Thr¹⁷² (Fig. 3B). However, unlike the activation of Akt, this response was unaffected by the tyrosine kinase inhibitor.

Under static conditions neither PP1 nor the phosphoinositide 3-kinase (PI3K) inhibitor, wortmannin, significantly affected eNOS activity as determined by monitoring intracellular cyclic GMP levels. However, both compounds abolished the shear-stress-induced accumulation of cyclic GMP (Fig. 3C). These data indicate that PP1-sensitive kinases are crucially involved in the activation of Akt and eNOS in response to fluid shear stress.

Effect of PECAM-1 knockdown on the shear-stress-induced activation of Akt and eNOS

To demonstrate a link between the shear-stress-induced activation of Src, the phosphorylation of PECAM-1 and the phosphorylation and activation of Akt and eNOS we determined the effects of decreasing PECAM-1 expression using siRNA oligonucleotides. The siRNA approach used markedly decreased the expression of PECAM-1 over 48 hours (to approximately 4% of the levels detected in untreated cells), without affecting the endogenous expression of β -catenin, β -actin (Fig. 4), eNOS, Akt, or AMPK (see Fig. 5).

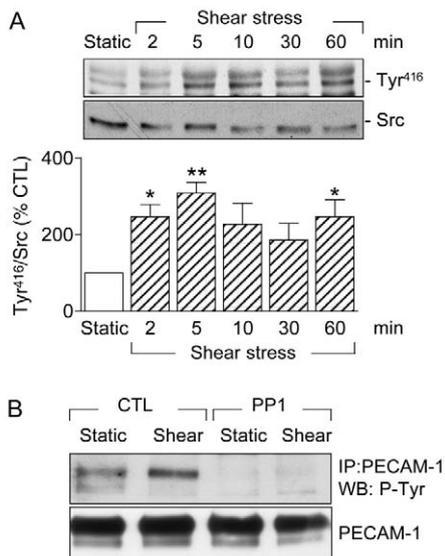


Fig. 2. Effect of shear stress on the activation of Src and phosphorylation of PECAM-1. (A) Endothelial cells were maintained under static conditions or exposed to fluid shear stress (12 dynes cm⁻², 5–60 minutes) and the phosphorylation of Src on Tyr⁴¹⁶ as well as total Src levels were detected using specific antibodies. The bar graph summarizes data obtained in four independent experiments; **P*<0.05, ***P*<0.01 versus static conditions. (B) Endothelial cells were exposed to fluid shear stress (12 dynes cm⁻², 30 minutes) in the absence (CTL) and presence of the Src kinase inhibitor PP1 (30 μ mol l⁻¹). Thereafter, PECAM-1 was immunoprecipitated and the tyrosine phosphorylation of PECAM-1 was assessed by western blotting. The results shown represent data obtained in three independent experiments.

The downregulation of PECAM-1 was associated with significant decreases in the shear-stress-induced phosphorylation of Akt and eNOS on Ser¹¹⁷⁷ (Fig. 5A,B). In none of the experiments performed did we observe either the complete inhibition of PECAM-1 expression or an abolition of the shear-stress-induced phosphorylation of eNOS or Akt. The PECAM-1 siRNA treatment did not affect the shear-stress-induced phosphorylation of AMPK (Fig. 5C).

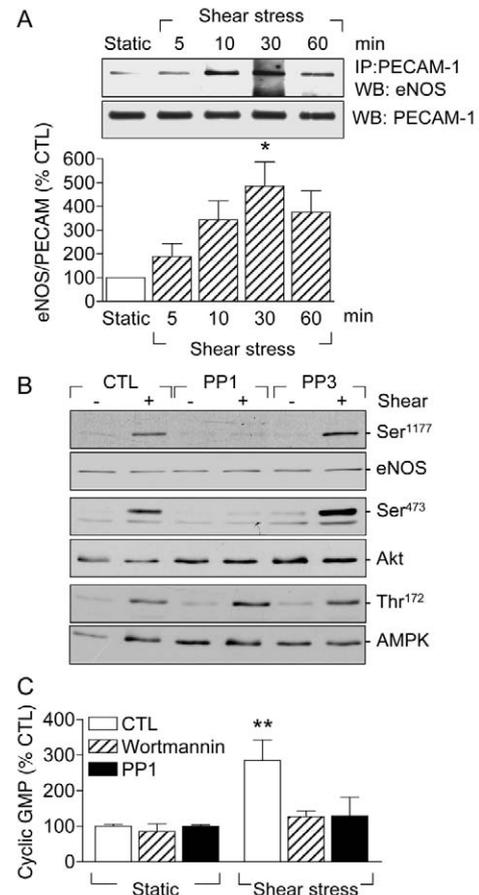


Fig. 3. Effect of shear stress on the association of eNOS with PECAM-1 and the phosphorylation of eNOS, Akt and AMPK and the generation of cyclic GMP. (A) Human endothelial cells were exposed to fluid shear stress (12 dynes cm⁻²) for the times indicated. Thereafter, PECAM-1 was immunoprecipitated and the co-precipitated eNOS was detected by a specific antibody. The bar graph summarizes data obtained in three independent experiments. (B) Human endothelial cells were exposed to fluid shear stress (12 dynes cm⁻², 30 minutes) in the absence (CTL) and presence of PP1 (30 μ mol l⁻¹) or its inactive analogue PP3 (30 μ mol l⁻¹). Thereafter, the Triton X-100-soluble cell fraction was subjected to SDS-PAGE and the phosphorylation of eNOS (Ser¹¹⁷⁷), Akt (Ser⁴⁷³) and AMPK (Thr¹⁷²) was determined using phospho-specific antibodies. The blots shown are representative of data obtained in three additional experiments. (C) Cells were incubated with solvent (CTL), wortmannin (40 nmol l⁻¹) or PP1 (30 μ mol l⁻¹) in the presence of IBMX (50 μ mol l⁻¹) and either maintained under static conditions or exposed to shear stress (12 dynes cm⁻², 30 minutes). Intracellular cyclic GMP levels were determined by radioimmunoassay and the bar graph summarizes data obtained in four independent experiments (each performed in duplicate); **P*<0.05, ***P*<0.01 versus static conditions.

To evaluate the functional consequences of the reduced phosphorylation of eNOS Ser¹¹⁷⁷ we assessed the shear-stress-induced activation of eNOS by monitoring intracellular cyclic GMP levels. In control (untreated) and scrambled siRNA-treated endothelial cells, shear stress elicited a time-dependent increase in cyclic GMP levels over 60 minutes. This effect was reduced in cells treated with the PECAM-1-specific siRNA (Fig. 6A). eNOS activation to receptor-dependent agonists was however unaffected by the downregulation of PECAM-1 as the siRNA procedure failed to affect the increase in cyclic GMP levels induced by a sub-maximal concentration of bradykinin (10 nmol l⁻¹) (Fig. 6B).

Comparison of the response to fluid shear stress in endothelial cells isolated from wild-type and PECAM-1^{-/-} mice

To test further the involvement of PECAM-1 in the shear-stress-induced activation of eNOS, we isolated endothelial

cells from the lungs of wild-type and PECAM-1^{-/-} mice. In endothelial cells from wild-type animals, shear stress (12 dynes cm⁻², 30 minutes) resulted in the phosphorylation of eNOS on Ser¹¹⁷⁷, as well as the phosphorylation of Akt and AMPK (Fig. 7). However, in endothelial cells from PECAM-1^{-/-} animals, the shear-stress-induced phosphorylation of eNOS and Akt was blunted. As in the experiments performed using the siRNA approach, the lack of PECAM-1 did not affect the shear-stress-induced phosphorylation of AMPK (Fig. 7).

The application of shear stress to endothelial cells from wild-type mice elicited an increase in cyclic GMP that was comparable to that detected in human endothelial cells (Fig. 8). Shear stress also stimulated an increase in cyclic GMP levels in cells from PECAM-1^{-/-} mice but this response was attenuated with respect to that observed in cells expressing PECAM-1.

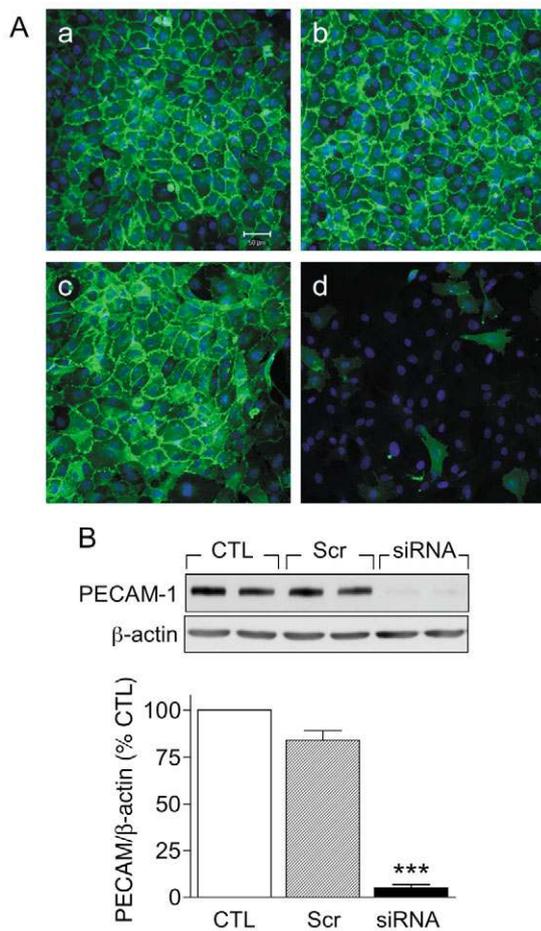


Fig. 4. Down regulation of PECAM-1 using siRNA. The expression of PECAM-1 was assessed by (A) immunofluorescence (PECAM-1=green; TOPRO-3=blue) and (B) western blotting using specific antibodies. Primary cultures of human endothelial cells were either left untreated (a, CTL) or treated with either (b) liposomal transfection agent, (c) scrambled oligonucleotides (Scr) or (d) PECAM-1 siRNA (siRNA) oligonucleotides for 48 hours. The bar graph summarizes the results of six independent experiments; ****P*<0.001 versus CTL.

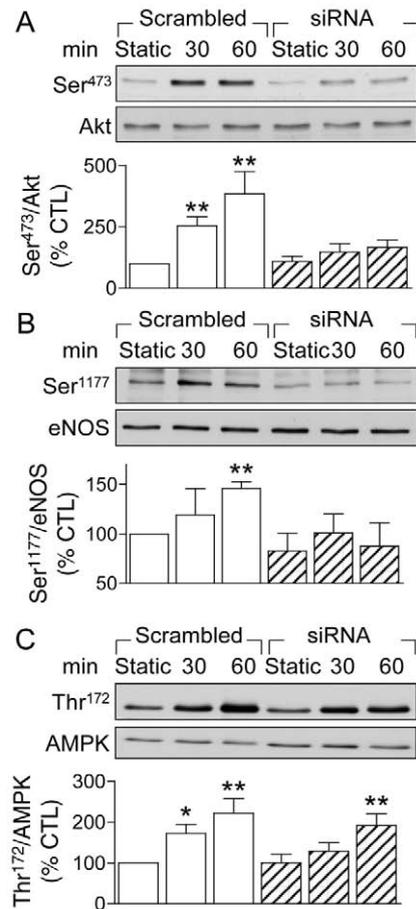


Fig. 5. Effect of PECAM-1 downregulation on the shear-stress-induced phosphorylation of Akt, eNOS and AMPK. Primary cultures of human endothelial cells treated with either scrambled oligonucleotides or siRNA against PECAM-1 were exposed to fluid shear stress (12 dynes cm⁻², 30 or 60 minutes) and the phosphorylation and expression of (A) Akt, (B) eNOS and (C) AMPK, were detected using specific antibodies. The western blots were quantified as phospho-protein per total protein and expressed relative to the control (CTL) conditions, i.e. the signal obtained in cells treated with scrambled oligonucleotides and maintained under static conditions. The bar graphs summarize the results of six independent experiments; **P*<0.05, ***P*<0.01 versus CTL.

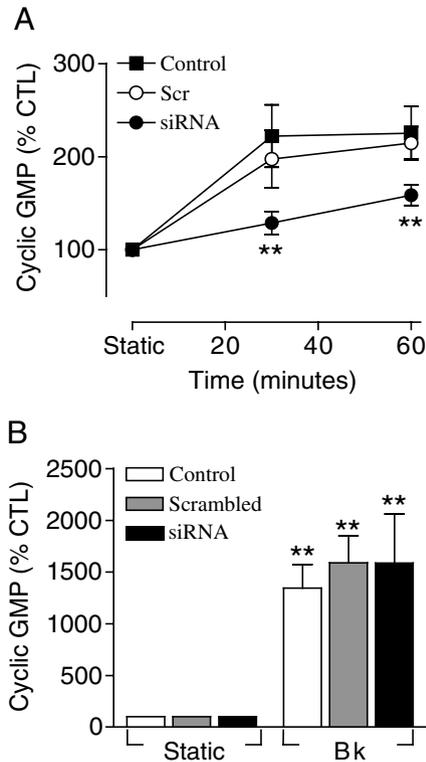


Fig. 6. Effect of PECAM-1 downregulation on the shear stress- and bradykinin-induced activation of eNOS. Human endothelial cells were left untreated (Control), treated with scrambled oligonucleotides (Scr) or siRNA against PECAM-1 and after 48 hours either (A) exposed to fluid shear stress (12 dynes cm^{-2}) or (B) maintained under static conditions (Static) and stimulated with bradykinin (Bk; 10 nmol l^{-1} , 5 minutes). The graphs presented summarize the results of four independent experiments each performed in triplicate; * $P < 0.05$, ** $P < 0.01$ versus static conditions.

Discussion

The results of the present study indicate that PECAM-1 is involved in the transduction cascade that translates the mechanical stimulation of endothelial cells into the activation of eNOS. Moreover, since PECAM-1 can modulate the shear-stress-induced phosphorylation of Akt and eNOS but not the shear-stress-induced activation of the AMPK it appears that there is a certain redundancy in the signaling pathways activated by the mechanical stimulation of endothelial cells.

The shear-stress-induced activation of eNOS is biphasic and consists of an initial Ca^{2+} -dependent peak followed by a plateau phase at a lower level that is largely independent of a maintained increase in intracellular Ca^{2+} (Kuchan and Frangos, 1994; Ayajiki et al., 1996). The secondary phase of NO production has been linked to the phosphorylation of the enzyme on Ser¹¹⁷⁷ and Ser⁶³³ by the kinases Akt (Dimmeler et al., 1999; Fulton et al., 1999) and PKA (Boo et al., 2002b; Boo et al., 2002a). Indeed, the phosphorylation of the enzyme on these sites increases basal NO production without the need of an increase in intracellular Ca^{2+} levels (McCabe et al., 2000; Boo et al., 2003). Links have previously been made between PECAM-1 and the PI3K/Akt pathway as PI3K can associate with PECAM-1 in neutrophils (Pellegatta et al., 1998) and in

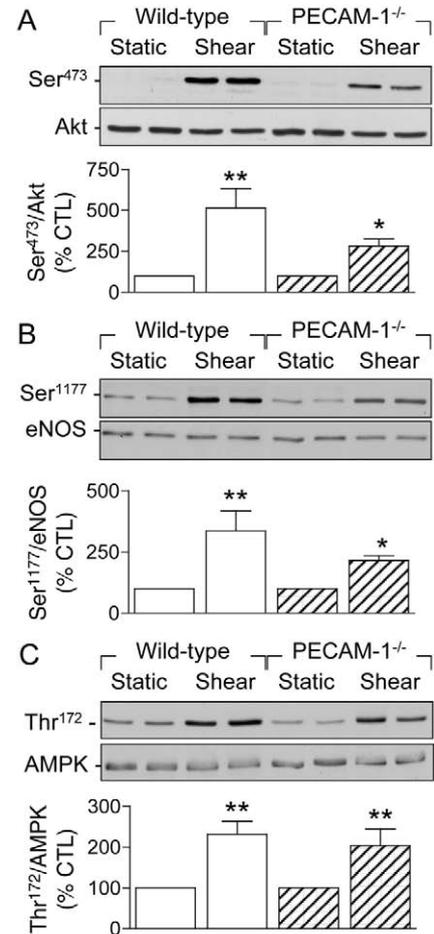


Fig. 7. Comparison of the shear-stress-induced phosphorylation of eNOS, Akt and AMPK in endothelial cells from wild-type and PECAM-1^{-/-} mice. Confluent cultures of lung endothelial cells from wild-type (WT) or PECAM-1^{-/-} mice were either maintained under static conditions or subjected to fluid shear stress (Shear; 12 dynes cm^{-2} ; 30 minutes). The phosphorylation of eNOS on Ser¹¹⁷⁷, Akt on Ser⁴⁷³ and AMPK on Thr¹⁷² was determined by western blotting with specific antibodies (duplicates are shown). The western blots were quantified as phospho-protein/total protein and expressed relative to the control (CTL) conditions, i.e. the signal obtained under static conditions. The bar graph summarizes data obtained in five independent experiments; * $P < 0.05$, ** $P < 0.01$ versus the response recorded in cells from wild-type mice.

a subset of CD14-positive circulating leukocytes where the ligation of PECAM-1 leads to the activation of Akt and to the induction of Bcl-2 and Bcl-X (Ferrero et al., 2003). In an immortalized PECAM-1-deficient mouse lung endothelioma cell line (Gratzinger et al., 2003), the phosphorylation of Akt is constitutively elevated (our unpublished observations). However, as the latter phenomenon could not be reversed by the reconstitution of PECAM-1 the constitutively high activation of Akt can be attributed to factors other than the lack of PECAM-1.

The activation of eNOS is regulated by a number of mechanisms and depends on the intracellular localization of the enzyme as well as its phosphorylation and the signaling molecules that comprise the eNOS signalosome (Fleming and

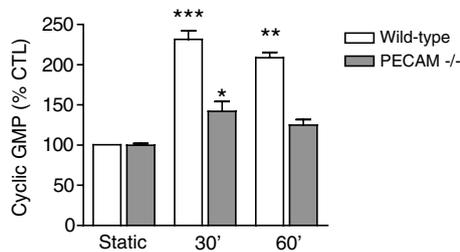


Fig. 8. Comparison of the shear-stress-induced increase in cyclic GMP in endothelial cells from wild-type and PECAM-1^{-/-} mice. Endothelial cells were incubated with IBMX (50 $\mu\text{mol l}^{-1}$) and either maintained under static conditions or exposed to shear stress (12 dynes cm^{-2}) for up to 60 minutes. Intracellular cyclic GMP levels were determined by radioimmunoassay and the bar graph summarizes data obtained in three independent experiments (each performed in duplicate); * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus static conditions.

Busse, 2003; Minshall et al., 2003). PECAM-1 and eNOS co-localize at the endothelial cell plasma membrane (Govers et al., 2002), and although there is considerable variation in the extent of this co-localization in different endothelial cells (Andries et al., 1998), PECAM-1 has been reported to physically associate with the eNOS complex (Dusserre et al., 2004). However, rather than the association of the two proteins increasing upon exposure to shear stress, as reported in this study, the association of eNOS with PECAM-1 decreased following cell stimulation (Dusserre et al., 2004). The reasons for these discrepant findings are currently unclear as we found comparable effects in primary cultures of human umbilical vein and porcine aortic (data not shown) endothelial cells but may be related to the time frame in which the experiments were performed. Indeed, the dissociation was reported to occur between 15 and 60 seconds after the application of shear stress (Dusserre et al., 2004), i.e. during the initial Ca^{2+} -dependent phase of the response. Here, we report an enhanced association of PECAM-1 and eNOS from 5 to 60 minutes after the application of shear stress and have otherwise concentrated on more delayed responses within the secondary Ca^{2+} -independent phase of the shear stress response. Both the conclusions of the above-mentioned study and our own results indicating that a physical association exists between eNOS and PECAM-1 are based on co-immunoprecipitation experiments but currently no information is available to indicate whether this interaction is direct or mediated by other adaptor or scaffolding proteins. Moreover, although alterations in the association of the two proteins have been temporally correlated to changes in cellular NO output, there is currently no direct evidence demonstrating that the activity of the PECAM-1-associated eNOS is any different to that of the disassociated enzyme.

The shear-stress-induced tyrosine phosphorylation of PECAM-1 is a delayed phenomenon relative to the activation of Ras (Gudi et al., 2003) or of K^+ channels (Olesen et al., 1988) and the fact that a tyrosine kinase must be activated in order for PECAM-1 to be phosphorylated means it is unlikely that PECAM-1 acts as a mechanoreceptor per se. Indeed, fluid shear stress elicits the tyrosine phosphorylation of PECAM-1 mutants that lack the transmembrane domain of the adhesion molecule and that do not localize to the lateral membranes or

participate in cell-cell homophilic PECAM-1 binding (Kaufman et al., 2004). Rather, it is more likely that PECAM-1 modulates endothelial cell activation in response to shear stress by virtue of its function as a scaffold for the binding of signaling molecules such as the tyrosine phosphatase SHP-2 and the scaffolding protein Gab-1 (for recent reviews see Newman and Newman, 2003; Ilan and Madri, 2003). Interestingly, Gab-1 translocates from the cytoplasm to endothelial cell junctions in response to flow (Osawa et al., 2002) and a dominant negative form of the non-receptor protein-tyrosine kinase, Fer; which can phosphorylate PECAM-1, SHP-2 and Gab-1 in vitro, is reported to prevent the engagement-dependent phosphorylation of PECAM-1 (Kogata et al., 2003). A role for Gab-1 in the shear-stress-induced phosphorylation and activation of Akt and activation of eNOS has also been recently reported (Jin et al., 2005) although in the latter study the Src-dependent phosphorylation of Gab-1 was linked to that of the vascular endothelial growth factor receptor 2 (VEGFR2) rather than to the tyrosine phosphorylation of PECAM-1.

During the preparation of this manuscript it was reported that ERK1/2 and Akt can be phosphorylated in shear stress-stimulated endothelial cells independently of the presence of PECAM-1 (Sumpio et al., 2005). Indeed, similar responses to shear stress were observed in human endothelial cells with and without PECAM-1 siRNA treatment as well as in murine PECAM-1^{-/-} endothelial cells with and without reconstitution of the protein. These observations are in direct contrast with those of the present study and although we also observed the phosphorylation of Akt in primary cultures of human endothelial cells treated with PECAM-1 siRNA as well as in endothelial cells from the lungs of PECAM-1^{-/-} mice, in our study the downregulation and/or complete loss of PECAM-1 was associated with a marked attenuation of the shear-stress-induced response. Interestingly, the downregulation of PECAM-1 (by ~96%) using the siRNA approach was more effective at attenuating shear-stress-induced phosphorylation of Akt and eNOS than its complete loss in cells from PECAM-1^{-/-} mice. We can only assume that developmental compensation occurs to atone for the lack of PECAM-1. Thus our data tend to support a role for PECAM-1 in the mechanotransduction signaling cascade in endothelial cells that is activated in response to shear stress.

While we have concentrated on a link between PECAM-1, Akt and eNOS, there are other kinases that are activated by fluid shear stress and that are able to phosphorylate and activate eNOS, the most important of which is probably PKA (Boo and Jo, 2003). Unfortunately we have currently no information regarding the role played by PECAM-1 and/or tyrosine kinases in the regulation of PKA activity in endothelial cells. The AMPK has also been reported to phosphorylate eNOS on Ser¹¹⁷⁷, and in certain conditions also on Thr⁴⁹⁵ (Chen et al., 1999). The present study is the first to report that the kinase can also be activated by exposing endothelial cells to fluid shear stress. The signaling events upstream of the activation of AMPK remain to be elucidated but our results indicate that these are distinct from those leading to the activation of Akt. Indeed, in contrast to Akt, the activation of AMPK by fluid shear stress was unaffected by the tyrosine kinase inhibitor PP1 as well as by the downregulation/lack of PECAM-1. As the AMPK is reported to be a redox-sensitive kinase (Zou et al.,

2002) and the endothelial production of reactive oxygen species is enhanced in response to shear stress (De Keulenaer et al., 1998; McNally et al., 2003), it is tempting to suggest that the activation of a superoxide-generating enzyme such as the NADPH oxidase or xanthine oxidase underlies this response.

Taken together, our data implicate PECAM-1 in the shear-stress-induced activation of Akt and eNOS at the same time as clearly indicating that this is not the only mechanotransduction pathway activated by shear stress in endothelial cells. Thus, the endothelial response to hemodynamic stimuli most likely reflects an integrated response of multiple signaling networks at different subcellular locations.

The authors are indebted to Isabel Winter, Tanja Megies and Tanja-Maria Mareczek for expert technical assistance and to Ruth Michaelis for performing the immunofluorescence studies. This study was supported by the Deutsche Forschungsgemeinschaft: SFB 553, B1. The authors of this manuscript belong to the European Vascular Genomics Network a Network of Excellence supported by the European Community's sixth Framework Program (Contract N° LSHM-CT-2003-503254).

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