Inhibition of Endothelial Nitric Oxide Synthase Activity by Proline-Rich Tyrosine Kinase 2 in Response to Fluid Shear Stress and Insulin

Beate Fisslthaler, Annemarieke E. Loot, Annisuddin Mohamed, Rudi Busse,† Ingrid Fleming

Abstract—In native and primary cultures of endothelial cells, fluid shear stress elicits the tyrosine phosphorylation of the endothelial NO synthase (eNOS), however, the consequences of this modification on enzyme activity are unclear. We found that fluid shear stress induces the association of eNOS with the proline-rich tyrosine kinase 2 (PYK2) in endothelial cells and that the eNOS immunoprecipitated from eNOS- and PYK2-overexpressing HEK293 cells was tyrosine-phosphorylated on Tyr657. In mouse carotid arteries, the overexpression of wild-type PYK2, but not a dominant-negative PYK2, decreased eNOS activity (∼50%), whereas in murine lung endothelial cells, the downregulation of PYK2 (small interfering RNA) increased ionomycin-induced NO production. Mutation of Tyr657 to the phosphomimetic residues aspartate (D) or glutamate (E) abolished enzyme activity, whereas a nonphosphorylatable mutant (phenylalanine [F]) showed activity comparable to the wild-type enzyme. Moreover, normal flow-induced vasodilatation was apparent in carotid arteries from eNOS−/− mice overexpressing either the wild-type eNOS or the Y657F mutant, whereas no flow-induced vasodilatation was apparent in arteries expressing the Y657E eNOS mutant. Insulin also activated PYK2 and stimulated eNOS in endothelial cells expressing the Y657F mutant but not wild-type eNOS. These data indicate that PYK2 mediates the tyrosine phosphorylation of eNOS on Tyr657 in response to fluid shear stress and insulin stimulation and that this modification attenuates the activity of the enzyme. The PYK2-dependent inhibition of NO production may serve to keep eNOS activity low and limit the detrimental consequences of maintained high NO output, ie, the generation of peroxynitrite. (Circ Res. 2008;102:1520-1528.)

Key Words: blood flow ■ insulin ■ mechanotransduction ■ nitric oxide ■ nitric oxide synthases ■ phosphorylation

Over the last 10 years, it has become clear that the endothelial nitric oxide (NO) synthase (eNOS) is regulated both by changes in the intracellular concentration of free Ca2+, as well as by the phosphorylation of the enzyme.1 Most is known about the role played by Ser1177 (human sequence), which is situated in the reductase domain of the enzyme and Thr495, located in the calmodulin (CaM)-binding domain, in the regulation of NO production, the phosphorylation of which appear to play a reciprocal role in the regulation of eNOS activity.2,3

There are several potentially phosphorylatable tyrosine residues in eNOS, and there have been numerous reports showing that tyrosine kinase inhibitors attenuate endothelial NO production and flow-induced vasodilatation.4–6 It is clear that the enzyme can be tyrosine-phosphorylated in endothelial cells treated with tyrosine phosphatase inhibitors,6,7 H2O2,7,8 or exposed to fluid shear stress,9 as well as in cells overexpressing v-Src.8,10 Indeed, Src was reported to phosphorylate a tyrosine residue (Tyr83, bovine sequence; Tyr81, human sequence) in the oxygenase domain of eNOS in bovine aortic endothelial cells. This modification was reported to be associated with an increase in NO production but because no differences in maximal eNOS activity were detected between the wild-type and the phenylalanine (Tyr81Phe) eNOS mutant, the authors proposed that tyrosine phosphorylation does not modify eNOS activity directly but may modulate the sensitivity of the enzyme to Ca2+, alter protein–protein interactions, or change its subcellular localization.8

Given that the above-mentioned study was performed using multipassaged cells overexpressing a specific tyrosine kinase and that we have previously only been able to detect endogenous eNOS tyrosine phosphorylation in primary cultures of endothelial cells,9 the aim of this study was to identify the amino acid(s) in eNOS that is tyrosine-phosphorylated following endothelial cell stimulation by physiological stimuli, as well as the consequences of this modification on enzyme activity. Because fluid shear stress is known to stimulate the activation of c-Src11 and the proline-rich tyrosine kinase (PYK2),12 we concentrated initially on the...
role of these kinases in the regulation of endothelial NO production.

### Materials and Methods

#### Materials

The specific eNOS antibody used for Western blotting or immunoprecipitation was from Santa Cruz Biotechnology (Heidelberg, Germany), the specific phospho-Tyr656 eNOS antibody was generated by Eurogentec (Searing, Belgium), and the anti-PYK2 from BD Biosciences (Heidelberg, Germany). The immunoprecipitation of tyrosine phosphorylated proteins was performed using a mixture of antibodies (from Cell Signaling, Santa Cruz Biotechnology, Transduction, and Millipore). N'-Nitro-l-arginine (L-NA), N'-nitro-l-arginine methyl ester (L-NAME), insulin, ionomycin, and all other substances were from Sigma (Deisenhofen, Germany).

#### Cell Culture and Transfection

Human umbilical vein cells, porcine aortic endothelial cells, and murine lung endothelial cells were isolated and cultured as described. Confluent cultures were transferred to culture medium containing 2% FCS and either maintained under static conditions or exposed to shear stress (12 dyne cm⁻²) in a cone-plate viscosimeter. The eluted proteins were concentrated, and the silver-stained protein band at 140 kDa was detected by immunoblotting as described.

#### In Vitro Phosphorylation

**Tyrosine Phosphorylation of eNOS by PYK2**

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**Immunoprecipitation and Immunoblotting**

eNOS, tyrosine-phosphorylated proteins, or PYK2 were immunoprecipitated with the appropriate antibodies. Detergent (Triton X-100)-soluble proteins or immunoprecipitates were heated with SDS-PAGE sample buffer and separated by SDS-PAGE, and specific proteins were detected by immunoblotting as described.

**Metabolic Labeling**

Porcine aortic endothelial cells were incubated in phosphate-free Tyrode’s solution containing H₃²PO₄ (Hartmann Analytic, Braunschweig, Germany; 30 μmol/L, 0.125 mcCi/mL, 12 hours) before the application of fluid shear stress (12 dyne cm⁻², 10 minutes) or insulin (10 nmol/L, 15 minutes). The immunoprecipitation of eNOS, SDS-PAGE, and phospho–amino acid detection was performed as described.

**Identification of the Phosphorylated Tyrosine Residue in eNOS**

HEK293 cells were cotransfected with eNOS and PYK2 or Src plasmids. After 48 hours, the cells were lysed with a hypotonic lysis buffer (5 mmol/L Tris-HCl, pH 7.4, 2 mmol/L EDTA with protease inhibitors, 2 mmol/L orthovanadate and 10 mmol/L okadaic acid). The membrane fraction was isolated by centrifugation, and NADP-binding proteins were purified by application of the supernatant to an ADP–sepharose column. The eluted proteins were concentrated, suspended in radioimmunoprecipitation assay buffer, and tyrosine phosphorylated proteins were immunoprecipitated with a mixture of phospho-tyrosine–specific antibodies. Immunoprecipitates were separated by SDS-PAGE, and the silver-stained protein band at 140 kDa was excised for further analysis (tryptic digest, immobilized metal-affinity chromatography and matrix-assisted laser desorption ionization–mass spectrometry [MALDI-MS]) by TopLab (Martinsried, Germany).

**Small Interfering RNA**

PYK2 gene expression silencing in mouse endothelial cells was achieved by transfection of small interfering (si)RNA duplexes (Stealth Select RNAi, Invitrogen, Paisley, UK) with TransGene II reagent (MoBiTec, Goettingen, Germany) as described. As a negative control, cells were treated with negative universal control oligonucleotides with medium GC content (Invitrogen).

**Measurement of NO Formation and eNOS Activity**

HEK293 cells were cotransfected with eNOS and either LacZ, c-Src, or PYK2 expression plasmids. The formation of NO in the transfected cells was determined by electron spin resonance spectroscopy using DETC as spin trap. The activity of the eNOS in the microsomal protein fraction of eNOS-overexpressing HEK293 cells was determined by the conversion of H²-l-arginine to l-citrulline as described.

**Determination of cGMP Concentration**

Cultured lung endothelial cells from wild-type or eNOS⁻/⁻ mice were infected with eNOS-overexpressing adenoviruses or treated with siRNA, suspended by treatment with Accutase, and washed in HEPES-buffered Tyrode solution containing the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) (1 mmol/L) and superoxide dismutase (100 U/mL). Approximately 5×10⁵ cells were added to RFL6 fibroblasts cultured in 24-well plates and incubated (37°C) in the absence or presence of L-NA, insulin (1 μmol/L, 30 minutes), or ionomycin (100 nmol/L, 2 minutes). Thereafter, the incubation was stopped by the addition of trichloroacetic acid (6%), and the concentration of cGMP was determined by a specific radioimmunoassay (GE Healthcare Buchler GmbH, Braunschweig, Germany).

**Plasmids and Generation of Recombinant Adenoviruses**

Myc-tagged human eNOS cDNA (GenBank accession no. NM_000603) in pcDNA3.1myc/His was used for in vitro mutagenesis using the QuickChange XL site-directed mutagenesis kit (Stratagene, Amsterdam, Netherlands) with the oligonucleotides (mutated tyrosine codon indicated in bold letters) 5'-GGCTCCCGGGGCAAGCCTCACTGTG-3' (sense Y-F); 5'-GGCTCCCGGGGCAAGCCTCACTGGT-3' (sense Y-D); 5'-GGCTCCCGGGGCAAGCCTCACTCTCTG-3' (sense Y-E) in combination with the corresponding antisense primers. pAdShuttle-CMV plasmids containing the wild-type human PYK2 (GenBank accession no. BC033651) or K457M PYK2, as well as the wild-type and Y657D, Y657E, and Y657F eNOS mutants, were generated using the pAdEasy system (kindly provided by Dr Bert Vogelstein, Baltimore, Md), and the viruses were generated as described.

**Adenoviral Transduction of Endothelial Cells**

Subconfluent endothelial cell cultures were infected with adenoviruses overexpressing PYK2 wild-type or the point mutants described above or eNOS wild-type or eNOS Y657 point mutants as described.

**Adenoviral Infection of Mouse Carotid Arteries**

Male C57BL/6 or eNOS⁻/⁻ mice, 6 to 9 weeks of age (Charles River, Sulzfeld, Germany) were anesthetized with isoflurane. The carotid arteries were perfused with saline solution, partially freed of connective tissue, and incubated with virus solution (3×10⁹ pfu in 20 μL). Flow-induced vasodilatation was assessed 40 to 44 hours after infection as described.

**In Vitro Phosphorylation**

PYK2 was overexpressed in HEK293 cells, then immunoprecipitated from Triton X-100 soluble proteins, and resuspended in kinase reaction buffer (40 mmol/L HEPES, pH 7.0; 80 mmol/L NaCl; 8% glycerine; 0.8 mmol/L EDTA; 0.8 mmol/L dithiothreitol; 5 mmol/L MgCl₂). The kinase reaction contained 5 μCi of either 1 of the peptides (corresponding to human eNOS amino acids 647 to 666) biotinNH-CVFGGLSRAYPHFCARAFAV-COOH (Y657) or biotinNH-CVFGGLSRAYPHFCARAFAV-COOH (Y657F) as substrate and 2 μCi γ⁻³²P-ATP. After 30 minutes at 30°C, the reaction was separated by 16.5% Tris/Tricine PAGE (cathode buffer: 0.1
mol/L Tris; 0.1 mol/L Tricine; 0.1% SDS; anode buffer: 0.2 mol/L Tris/HCl, pH 8.9). The gel was extensively washed, and the peptides and proteins were visualized by silver staining. After drying, the gel was exposed to a x-ray film. The bands were excised, and the radioactive incorporation was quantified by scintillation counting.

Statistics
Data are expressed as means±SEM. Statistical evaluation was performed with Student’s t test for unpaired data and 1-way ANOVA followed by a Bonferroni t test or ANOVA for repeated measures where appropriate. Values of *P<0.05 were considered statistically significant.

Results
Role of PYK2 in the Tyrosine Phosphorylation of eNOS
eNOS immunoprecipitated from primary cultures of porcine aortic endothelial cells maintained under static conditions was phosphorylated on serine and threonine residues, whereas no consistent signal corresponding to the phosphorylation of tyrosine residues was detectable (Figure 1A). Consistent with our previous report,9 exposure of endothelial cells to fluid shear stress (12 dyne cm⁻²) for 10 minutes, and phospho-amino acid analysis of the immunoprecipitated eNOS was performed. The term “peptides” indicates nonhydrolyzed peptides; P-Y, phospho-tyrosine; P-T, phospho-threonine; P-S, phospho-serine; P_i, inorganic phosphate. B and C, Porcine endothelial cells were exposed to fluid shear stress for up to 120 minutes, and the association of PYK2 with the immunoprecipitated (IP) eNOS (B), as well as the association of eNOS with the immunoprecipitated PYK2 (C), was assessed by Western blotting (WB). D, HEK293 were cotransfected with eNOS and either LacZ, PYK2, or Src. Thereafter, either tyrosine phosphorylated proteins or eNOS were immunoprecipitated, and the Western blots were incubated with eNOS-specific or phospho-tyrosine-specific antibodies. The bar graph summarizes data obtained in 6 to 10 separate experiments. *P<0.05, **P<0.01 vs static or LacZ.

was only readily detectable in primary and first passaged porcine endothelial cells.

There was a weak association (comununoprecipitation) of PYK2 with eNOS in the cells maintained under static conditions (Figure 1B). However the association of PYK2 with eNOS increased following the application of shear stress and the 2 proteins remained associated as long as the stimulus was applied (up to 120 minutes). Shear stress also increased the association of eNOS with immunoprecipitated PYK2 (Figure 1C). PYK2 could not be immunoprecipitated with IgG (see Figure I in the online data supplement, available at http://circres.ahajournals.org). Again, this was a phenomenon that was readily demonstrable in primary/first passage cultures of porcine endothelial cells but not at higher passages because of the rapid decrease in PYK2 expression in the porcine endothelial cells studied. The expression of PYK2, however, did not decrease with time in culture in murine lung endothelial cells (see supplemental Figure II).

Because shear stress elicits the activation of both Src and PYK2, we assessed the ability of both kinases to phosphorylate eNOS when overexpressed in HEK293 cells. Both kinases elicited the phosphorylation of eNOS, as deter-
mined by the immunoprecipitation of tyrosine phosphorylated proteins and Western blotting for eNOS or vice versa (Figure 1D).

**Effect of PYK2 Overexpression on the Activity of eNOS**

We next determined the consequences of PYK2 and Src overexpression on the activity of eNOS when overexpressed in HEK293 cells. Using electron spin resonance spectroscopy, we observed that the overexpression of Src had little or no effect on the generation of NO, whereas the overexpression of PYK2 significantly decreased basal NO production (Figure 2A).

To ensure that a similar relationship exists in native endothelial cells, we overexpressed either PYK2 or a dominant-negative PYK2 mutant (K457M) in carotid arteries from wild-type mice before assessing flow-induced vasodilation. We found that whereas arteries expressing K457M PYK2 responded normally to stepwise increases in flow, and were sensitive to NOS inhibition, the overexpression of PYK2 essentially abrogated flow-induced, NO-mediated vasodilatation (Figure 2B). The residual NO-independent flow-induced vasodilatation was attributed to the endothelium-derived hyperpolarizing factor. Similarly, acetylcholine (1 μmol/L) elicited the relaxation of vessels overexpressing the wild-type PYK2, whereas no relaxation was observed in vessels overexpressing wild-type PYK2 (Figure 2C). Thus, in both the in vitro system and endothelial cells in situ, the overexpression of PYK2 was linked to attenuated NO output.

**PYK2 Phosphorylates eNOS on Tyr657**

To identify the tyrosine residue in eNOS phosphorylated by PYK2, we overexpressed both proteins in HEK293 cells, enriched the eNOS using ADP sepharose, and further enriched the tyrosine phosphorylated eNOS by immunoprecipitation with phospho-tyrosine antibodies before subjecting the samples to SDS–gel electrophoresis. The isolated eNOS protein was treated with iodoacetamide and digested with trypsin. Using MALDI-MS, a peptide with a mass of 1321 Da was detectable under control conditions and was absent after alkaline phosphatase treatment. The mass of this peptide matches a tryptic peptide from human eNOS with the amino acid sequence AYPHFCAFAR, which corresponds to residues 656 to 665 in the eNOS protein, thereby identifying Tyr657 as the tyrosine residue phosphorylated by PYK2.

We next assessed the ability of PYK2 immunoprecipitated from HEK293 cells to phosphorylate a small peptide corresponding to the appropriate eNOS sequence. Whereas PYK2 elicited the phosphorylation of the control peptide, no phosphorylation was observed when the tyrosine residue corresponding to Tyr657 was replaced by phenylalanine (Figure 3A). Essentially, the same results were obtained using point mutants of eNOS overexpressed in HEK293 cells, and whereas eNOS was clearly tyrosine phosphorylated in cells coexpressing the wild-type eNOS and either Src or PYK2 (see supplemental Figure III for demonstration of kinase expression), no tyrosine phosphorylation could be detected in cells coexpressing eNOS and K457M PYK2 or in cells expressing PYK2 together with an eNOS in which Tyr657 was replaced with either aspartate (Y657D) or glutamate (Y657E) to mimic tyrosine phosphorylation (Figure 3B). A weak signal was, however, detected when Tyr657 was replaced by the nonphosphorylatable amino acid phenylalanine (Y657F). Similar experiments were repeated using an antibody that selectively recognized the phosphorylated form of Tyr657. We observed Tyr657 eNOS phosphorylation in cells overexpressing PYK2 but not the Y657D, Y657E, or Y657F eNOS mutants. Moreover, no phosphorylation of wild-type eNOS could be detected in cells overexpressing the kinase-inactive PYK2 (Figure 3C; see supplemental Figure III for demonstration of kinase expression). Using the same antibody, it was possible to demonstrate that the application of shear stress to murine endothelial cells resulted in an ≈3-fold increase in the
Phosphorylation of eNOS on Tyr657 (Figure 3D). Murine instead of porcine cells were chosen for these experiments as the expression of PYK2 was maintained in passaged cells.

**Mimicking the Tyrosine Phosphorylation of Tyr657 Results in a Loss of eNOS Activity**

eNOS activity, as well as its sensitivity to Ca\(^{2+}\) and CaM, was assessed in lysates from HEK293 cells overexpressing either the wild-type enzyme or 1 of the Y657F, Y657D, or Y657E eNOS mutants. Whereas the wild-type enzyme and the nonphosphorylatable Y657F mutant displayed similar maximal activity, as well as sensitivity to Ca\(^{2+}\) and CaM, the Y657E and Y657D mutants failed to generate L-citrulline under any of the experimental conditions used (Figure 4A).

The results of this in vitro assay were then confirmed in cultured endothelial cells. Because cultured murine endothelial cells possess low guanylyl cyclase activity, we performed a transfer bioassay in which cGMP was assayed in RFL6 fibroblasts incubated with endothelial cells from eNOS\(^{-/-}\) mice that were either infected with the control virus or with viruses encoding wild-type eNOS, Y657E, or Y657F eNOS. As expected, basal and ionomycin-stimulated cGMP levels were significantly higher in fibroblasts incubated with endothelial cells expressing either the wild-type eNOS or the Y657F mutant than in cells treated with control virus. On the other hand, cGMP levels were similar in cells treated with cells lacking eNOS or expressing the Y657E eNOS mutant. Neither of the latter endothelial cells responded to ionomycin with an increase in NO production (Figure 4B). We were unable to detect any generation of superoxide anions by cells overexpressing the phosphomimetic mutants, indicating that the lack of NO production was not related to the uncoupling of the enzyme (data not shown).

Flow-induced, NO-dependent vasodilatation was not observed in carotid arteries from eNOS\(^{-/-}\) mice either under basal conditions (data not shown) or following overexpression of green fluorescent protein (Figure 5A). A significant flow-induced and NO inhibitor–sensitive vasodilatation was, however, observed in arteries overexpressing wild-type eNOS or the Y657F mutant, although the responses were slightly attenuated when compared with responses observed in arteries from wild-type mice (compare with Figure 2B). However, no flow-induced, NO-dependent vasodilatation was detectable in arteries expressing Y657E eNOS (Figure 5B).

**The Inability of Insulin to Stimulate NO Production in Endothelial Cells Can Be Accounted for by the PYK2-Dependent Phosphorylation of Tyr657**

In porcine aortic endothelial cells, insulin (10 nmol/L, 15 minutes) elicited the tyrosine phosphorylation of eNOS, as determined by 2D phospho–amino acid analysis (Figure 6A). The stimulation of murine endothelial cells with insulin (1 \(\mu\)mol/L, 15 minutes) also elicited a 257±57% increase \((n=6; P<0.05\) in the phosphorylation of eNOS on Tyr657, the association of the tyrosine-phosphorylated eNOS with PYK2 (Figure 6B), and the tyrosine phosphorylation of PYK2 (Figure 6C), as demonstrated by immunoprecipitation and Western blotting. Insulin stimulated the phosphorylation of eNOS on Ser1177 without exerting any consistent effect on the phosphorylation of Thr495 (supplemental Figure IV). We were unable to detect any consequence of the mutation of Tyr657 on the insulin-induced phosphorylation of either residue.

To determine whether eNOS tyrosine phosphorylation could affect the ability of insulin to stimulate the enzyme we
assessed the ability of insulin to increase cGMP levels in HEK293 cells expressing either wild-type eNOS or the Y657F eNOS mutant. cGMP levels were 8.6±0.5 versus 9.9±0.2 fmol/μg protein in the presence of solvent and insulin (1 μmol/L, 5 minutes) in HEK293 cells overexpressing the wild-type eNOS and the Y657F eNOS mutant respectively (n=3, P<0.05). We then assessed the consequences of downregulating PYK2 in mouse lung endothelial cells using siRNA on the ability of insulin to stimulate NO/cGMP production in murine endothelial cells. In cells treated with transfection agent (GTII) alone, insulin (1 μmol/L, 30 minutes) failed to increase cGMP levels, whereas ionomycin (0.1 μmol/L, 2 minutes) elicited a 2.5-fold increase that was abrogated by L-NA. Essentially the same results were obtained using a control siRNA. The downregulation of PYK2, however, slightly increased basal cGMP production, as well as the response to ionomycin, and unmasked the stimulatory effects of insulin on NO production (Figure 7).

Discussion

The results of this study demonstrate that fluid shear stress elicits the association of PYK2 with eNOS in endothelial cells expressing either wild-type eNOS or the Y657F eNOS mutant. cGMP levels were 8.6±0.5 versus 9.9±0.2 fmol/μg protein in the presence of solvent and insulin (1 μmol/L, 5 minutes) in HEK293 cells overexpressing the wild-type eNOS and the Y657F eNOS mutant respectively (n=3, P<0.05). We then assessed the consequences of downregulating PYK2 in mouse lung endothelial cells using siRNA on the ability of insulin to stimulate NO/cGMP production in murine endothelial cells. In cells treated with transfection agent (GTII) alone, insulin (1 μmol/L, 30 minutes) failed to increase cGMP levels, whereas ionomycin (0.1 μmol/L, 2 minutes) elicited a 2.5-fold increase that was abrogated by L-NA. Essentially the same results were obtained using a control siRNA. The downregulation of PYK2, however, slightly increased basal cGMP production, as well as the response to ionomycin, and unmasked the stimulatory effects of insulin on NO production (Figure 7).

Figure 4. eNOS mutants that mimic the phosphorylation of Y657 are inactive. A, [3H]-citrulline production in HEK293 cells expressing either wild-type eNOS or 1 of the Y657D, Y657E, or Y657DF eNOS mutants. Experiments were performed in the presence of varying concentrations of Ca2+ (0.1 to 1 μmol/L) and in the absence (open bars) or presence of (0.1 to 3 μmol/L) CaM. The inset shows comparable expression of the eNOS proteins in each group. B, Lung endothelial cells from eNOS+/− mice were infected with either a control virus or adenoviruses encoding wild-type eNOS or 1 of the Y657 eNOS mutants. Experiments were performed in the presence of solvent (S), ionomycin (I) (100 nmol/L, 2 minutes), and L-NA (300 μmol/L, 60 minutes), and NO bioavailability was assessed in a transfer bioassay by determining cGMP production in detector RFL6 fibroblasts by radioimmunoassay. The graphs summarize data from 6 to 9 separate experiments. *P<0.05, **P<0.01, ***P<0.001 vs solvent or L-NAME.

Figure 5. Effect of the eNOS mutants and PYK2 overexpression in isolated mouse carotid arteries. Carotid arteries from eNOS+/− mice were infected with adenoviruses encoding green fluorescent protein (GFP) or wild-type (WT) eNOS (A) and Y657E or Y657F eNOS (B). After 40 to 44 hours, flow-induced vasodilatation was assessed in the absence or presence of L-NAME (300 μmol/L). The Western blot demonstrates equal expression levels of the different eNOS mutants. The graphs summarize data from 6 to 9 separate experiments. *P<0.05 vs L-NAME.
cells and the tyrosine phosphorylation of the enzyme on Tyr657 within the FMN binding domain. The latter modification was linked to the inhibition of NO production and a marked attenuation of agonist- and flow-induced vasodilatation without any change in the generation of reactive oxygen species. Thus, the tyrosine phosphorylation of eNOS on Tyr657 in endothelial cells exposed to fluid shear stress serves to limit NO output.

Fluid shear stress is known to elicit the activation of c-Src, Src kinase inhibitors abrogate the activation of eNOS in response to shear stress, and c-Src can be coprecipitated with eNOS from bovine aortic endothelial cells. Moreover, the overexpression of v-Src and eNOS in COS-7 cells has recently been reported to increase enzyme activity, as well as to result in the phosphorylation of eNOS on Tyr81 (Tyr83 bovine sequence). Given the latter report, it was unexpected that a very similar approach, ie, the overexpression of eNOS and c-Src in HEK293 cells was without any significant effect on NO production, even though eNOS was clearly tyrosine phosphorylated. In contrast, the overexpression of eNOS with PYK2 clearly attenuated NO production, indicating that this kinase may play a more pronounced role than c-Src in the regulation of endothelial NO production.

PYK2 is a rather unusual tyrosine kinase in that it contains no Src homology (SH)2 or SH3 domains, has been implicated in regulating the organization of the actin cytoskeleton, and can be activated by integrin stimulation (for review see elsewhere). Although PYK2 was initially characterized as a Ca2+/H11001-dependent tyrosine kinase and has been shown to be regulated by extracellular Ca2+/H11001 influx in several different cell types, whether or not Ca2+ alone, without concomitant integrin activation, increases kinase activity in endothelial cells remains controversial. At first glance, there seems to be a conflict between the Ca2+/H11001 dependence of PYK2 and the Ca2+ independence of shear-stress-induced NO production; however, Ca2+ does not activate PYK2 in vitro assays and the kinase does not contain any known consensus sequences for Ca2+ binding, indicating that Ca2+ may be indirectly affecting PYK2 activity.

Our finding that eNOS in HEK cells was phosphorylated by PYK2 on Tyr657 was confirmed by in vitro peptide phosphorylation, as well as by site-directed mutagenesis of eNOS. Indeed, the mutation of Tyr657 to either aspartate or glutamate abrogated the tyrosine phosphorylation of the enzyme, whereas the replacement of Tyr657 with phenylalanine significantly attenuated eNOS phosphorylation, leaving only a weak phospho-tyrosine signal. Because no phosphorylation of the Y657F eNOS mutant could be detected using an anti-phospho-Tyr657 antibody, it would appear that Tyr657 is not the only tyrosine residue that can be phosphor-

Figure 6. Insulin elicits the tyrosine phosphorylation of eNOS and PYK2. A, 32P-labeled porcine aortic endothelial cells were incubated with solvent or insulin (10 nmol/L, 15 minutes), and phospho–amino acid analysis of the immunoprecipitated eNOS was performed. The term “peptides” indicates nonhydrolyzed peptides; P-Y, phospho-tyrosine; P-T, phospho-threonine; P-S, phospho-serine; P_i, inorganic phosphate. B and C, Murine lung endothelial cells were incubated with solvent or insulin (1 μmol/L, 15 minutes). Treated, either phospho-Tyr657 (pY), eNOS, total eNOS, or PYK2 were immunoprecipitated, and protein recovery, association, and phosphorylation were assessed by Western blotting. The bar graph summarizes data from 6 separate experiments. *P<0.05 vs control (CTL).

Figure 7. PYK2 siRNA sensitizes eNOS to insulin. Lung endothelial cells from wild-type mice were treated with either transfection agent alone (GTII), a control (CTL) siRNA, or siRNA directed against PYK2 and, after 48 hours, treated with solvent (Basal), insulin (1 μmol/L), ionomycin (0.1 μmol/L, 2 minutes), or L-NA (300 μmol/L, 60 minutes). NO bioavailability was assessed in a transfer bioassay by determining cGMP production in detector RFL6 fibroblasts by radioimmunoassay. The PYK2 Western blot demonstrates the effectiveness of the PYK2-directed siRNA in the mouse lung endothelial cells. The bar graphs summarize data from 5 to 9 separate experiments. *P<0.05, **P<0.01, ***P<0.001 vs basal.
Tyrosine Phosphorylation of eNOS by PYK2

Fisslthaler et al

1527

Tyrosine phosphorylation of eNOS by PYK2.

The consequence of eNOS Tyr657 phosphorylation was not, as expected, a subtle modification of NO production or the Ca2+ sensitivity of the enzyme but a complete loss of enzyme activity. However, a clue to why the mutation of this tyrosine residue could have such dramatic effects can be found by considering the mechanisms known to regulate the activity of the neuronal NOS, which was recently reported to be determined by a large-scale swinging motion of the FMN domain to deliver electrons to the catalytic module in the holoenzyme. From the crystal structure of neuronal NOS, the phosphorylation of a tyrosine residue (Tyr889, rat neuronal NOS sequence), which is in the vicinity of the FMN domain, could prevent its movement, essentially locking the FMN domain into its electron-accepting position, thus inhibiting enzyme activity. Because Tyr657 is the equivalent tyrosine residue in the human eNOS sequence, it is highly likely that its phosphorylation would be associated with a loss of NO production.

Perhaps the most physiologically intriguing observation made during the course of this study relates to the sensitivity of eNOS to insulin. We have repeatedly found that although the stimulation of native or primary cultures of endothelial cells with insulin results in the rapid phosphorylation of Akt, as well as eNOS on Ser1177, it fails to activate eNOS and increase endothelial cGMP levels or elicit relaxation. Given that the phosphorylation of Tyr657 inhibited eNOS activity, we hypothesized that the insulin-induced activation of PYK2 and tyrosine phosphorylation of Tyr657 may functionally antagonize the effects of the Ser1177 phosphorylation. Indeed, we found that insulin stimulated the tyrosine phosphorylation of both PYK2 and eNOS in murine endothelial cells and that the downregulation of PYK2 (using siRNA) rendered eNOS sensitive to insulin. At this stage, it should be noted, however, that it is impossible to rule out that the phosphorylation of others may functionally antagonize the effects of the Ser1177 phosphorylation.

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Disclosures

None.

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Online supplementary data

**Online Figure I.** Association of eNOS with PYK2. Porcine aortic endothelial cells were exposed to fluid shear stress for 30 minutes. Cell lysates were then incubated with either an IgG or with an eNOS antibody and the presence of PYK2 and eNOS in the immunoprecipitates was assessed by Western blotting.

**Online Figure II.** PYK2 expression in cultured porcine aortic endothelial cells versus mouse lung endothelial cells. (A) Porcine aortic endothelial cells were isolated and maintained under static conditions. PYK2 expression was determined by Western blotting in confluent cultures of primary or first passage (P1) endothelial cells. The expression of eNOS was also determined to demonstrate that other endothelial proteins were not affected by culture as well as the equal loading of each lane. (B) Expression of PYK2 in different passages (P2 to P7) of mouse lung endothelial cells; pc = HUVEC as positive control. Similar results were obtained in 2 additional experiments.
Online Figure III. Expression of Src and PYK2. Western blots from the HEK 293 lysates used for the immunoprecipitation of eNOS shown in Figure 3B and 3C demonstrating equal expression of PYK2, Src and β-actin.

Online Figure IV. Effect of the mutation of Tyr657 on the insulin-induced phosphorylation of eNOS on Ser1177 and Thr495 in HEK293 cells. Western blots from HEK 293 cells overexpressing either the wild-type (WT) eNOS or the Y657F or Y657D eNOS mutant. Cells were stimulated with insulin (1 or 3 µM; 15 minutes) and the phosphorylation of the enzymes on serine 1177 (pSer1177), threonine 495 (pThr495) was assessed using appropriate phospho-specific antibodies.