Glucose 6-Phosphate Dehydrogenase Is Regulated Through c-Src–Mediated Tyrosine Phosphorylation in Endothelial Cells

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Objective—Glucose 6-phosphate dehydrogenase (G6PD) maintains cellular NADPH levels, which are essential for cellular functions, such as vascular endothelial growth factor (VEGF)-induced angiogenesis. The molecular mechanisms regulating G6PD in angiogenesis are not fully understood. Because tyrosine phosphorylation is a key regulatory pathway for VEGF-mediated endothelial cell (EC) responses, we investigated tyrosine phosphorylation of G6PD and the role of the nonreceptor tyrosine kinase Src.

Methods and Results—VEGF increased G6PD membrane translocation as measured by a plasma membrane sheet assay, whereas tyrosine kinase inhibitor PP2 (4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo [3,4-d]pyrimidine) decreased G6PD translocation by 100%. Furthermore, G6PD tyrosine phosphorylation and plasma membrane activity were increased by VEGF. In resting ECs, tyrosine kinase inhibitors PP2 and herbimycin A decreased basal G6PD activity by \( \frac{1}{2} \), whereas transfection with kinase inactive Src (kDa-Src) decreased basal activity by \( \frac{1}{3} \). In mouse embryonic fibroblasts, Src-deficient (SYF) cells showed \( \frac{1}{3} \) lower basal G6PD activity than Src-expressing SYF cells. In addition, Src directly phosphorylated G6PD assayed by in vitro kinase assay. In ECs transfected with the G6PD mutants Y428F, Y507F (presumptive sites for Src-phosphorylation) or double mutant Y428F/Y507F, G6PD tyrosine phosphorylation was significantly decreased. Finally, G6PD tyrosine mutants (Y428F, Y507F, and Y428F/Y507F) decreased VEGF-mediated Akt phosphorylation and EC migration.

Conclusions—G6PD activity and membrane association are regulated by Src-mediated tyrosine phosphorylation, which contributes to VEGF-mediated cellular responses in EC. (Arterioscler Thromb Vasc Biol. 2009;29:00-00.)

Key Words: G6PD  ■  Src  ■  VEGF  ■  endothelial cells  ■  tyrosine phosphorylation

The importance of G6PD as an antioxidant enzyme has been well recognized. Targeted deletion of G6PD in embryonic stem cells showed that it was essential for maintenance of cellular redox homeostasis. In humans, G6PD deficiency is associated with neonatal jaundice, drug- and infection-mediated hemolysis, favism, and chronic nonspherocytic hemolytic anemia.

Recent studies have demonstrated that altered G6PD activity affects diverse physiological functions including cell growth and signaling, embryonic development, and susceptibility to viral infection. In the cardiovascular system, G6PD modulates the contractile phenotype of cardiomyocytes and angiotensin II vasoreactivity. Deficiency of G6PD increases myocardial dysfunction after ischemia-reperfusion. In endothelial cells (ECs), G6PD regulates vascular tone through NO production and angiogenesis mediated by VEGF. However, the mechanisms by which cardiovascular agonists regulate G6PD are not fully understood.

We investigated the mechanisms by which G6PD is activated in response to VEGF. We demonstrate that G6PD translocates to the plasma membrane and is tyrosine phosphorylated by c-Src. Specifically, Src phosphorylates G6PD at Y428 and Y507, which are required for VEGF-induced Akt activation and EC migration.

Methods

Antibodies and Reagents

Antibodies

Actin was from Santa Cruz; G6PD was from Bethyl Laboratories, phospho-Akt (S473), and phospho-eNOS (S1177) were from Cell Signaling; Src (GD11) and phosphotyrosine (4G10) were from Upstate; Flag was from Roche.

Reagents

PP2 (4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine) was from EMD Bioscience; VEGF was from R&D. Human G6PD cDNA construct was a generous gift from Dr Veronica Lam (University of Hong Kong, Hong Kong). It was subcloned into FLAG-tagged pCMV2A vector (Stratagene) using the following primers: 5'-CCG GAA TTC CAT GGC AGA GGT GCC CCT-3', and 5'-CCG CTC GAG CGG AGC TTG TGG GGG

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TTC ACC C-3', kDa-Src (K295R) was a generous gift from Jonathan A. Cooper (University of Washington, Seattle).

Cell Culture and Plasmid Transfection

Bovine aortic endothelial cells (BAECs) were purchased from Clonetics and cultured in Medium 199 supplemented with 10% FETALCLONE III (Hyclone), basal MEM vitamins, and amino acids (Invitrogen). Cells of passages 6 to 10 were used for experiments. BAECs at >80% confluence were used for transfection. Lipofectamine 2000 (Invitrogen) was used according to the manufacturer’s instructions. Briefly, ECs were seeded in 6-well plates 1 day before transfection, then incubated with 2 μg of plasmids using 5 μL of lipofectamine 2000 for 4 hours. For VEGF stimulation, ECs serum starved for 24 hours were treated with 20 ng/mL VEGF for indicated time points. Then proteins were harvested for further analysis.

SYF cells were a generous gift from Jonathan A. Cooper (University of Washington, Seattle) and cultured in DMEM supplemented with 10% fetal bovine serum and 500 μg/mL G418.

In Vitro Kinase Assay

ECs were stimulated with VEGF (20 ng/mL), and Src was immunoprecipitated as described.15 The G6PD-GST fusion protein was incubated with immunoprecipitated Src in the presence of 10 μCi of [γ-32P]ATP (Amersham Bioscience), 100 μmol/L of ATP, and 15 mmol/L of MgCl2 for 30 minutes at 30°C in kinase assay buffer containing 20 mmol/L of MOPS, pH 7.2, 25 mmol/L β-glycerophosphate, 5 mmol/L EGTA, 1 mmol/L sodium orthovanadate, and 1 mmol/L dithiothreitol.

Site-Directed Mutagenesis

The QuikChange site-directed mutagenesis kit (Stratagene) was used to generate the G6PD mutations Y428F, Y507F, and G6PD-Y428F/Y507F. The primers used were: Y428F, 5’-ACC TAC GCC AAC AGA TTC AAG AAC GTG AAG CTC CCT-3’ and 5’-AGG GAG CTT CAC GTT CTG GTA GAA GGT GCC GTA GGA CTT-3’; Y507F, 5’-CAG TAT GAG GCC ACC TTC AAG TAG GTG GTC AAC CCC-3’ and 5’-GGG GTT CAC CCA CTG GAA GGT GCC CTC ATA CTG-3’. PCR reactions and transformation were performed following the instructions of the manufacturer.

Western Blot and Immunoprecipitation

Western blots and immunoprecipitations were performed as described previously.15 The resulting autoradiograms were analyzed with Image J (1.36b, National Institute of Health). Equal loading of protein was ensured by measuring actin expression.

G6PD Activity Assay

G6PD activity was assayed by the measurement of NADPH production in a microplate reader (Victor) following manufacturer’s instructions (Invitrogen).

Plasma Membrane Sheet Assay and Immunofluorescence

Preparation of plasma membrane sheets and immunofluorescence were performed as described previously with modifications.16,17 ECs grown on 35-mm dishes were growth arrested overnight by incubation in serum-free medium and stimulated with VEGF (20 ng/mL) for 6 mL of sonication buffer (1× KHMGE buffer supplemented with 10 mmol/L DTT and 1 mmol/L phenylmethylsulphonyl fluoride). The remaining plasma membrane sheets were washed 3 times with sonication buffer to remove any remaining cytosolic proteins. Immunostaining was performed using G6PD antibody.16,17 Fluorescent intensity was quantified with Image J (1.36b, National Institutes of Health).

EC Migration

For detection of cell migration, a wound-healing assay was performed.18,19 BAECs were grown to confluence in a 6-cm dish and continuously cultured in serum free medium for an additional 24 hours. A longitudinal wound was made in the middle of the dish with a sterile scalpel. Cells were stimulated with VEGF (20 ng/mL) for 6 hours, and cell migration was measured as percentage of recovered area using light microscopy and the computer program Image J (1.36b, National Institute of Health).

Statistical Analysis

Data are expressed as mean±SE. Comparisons between groups were performed by Student’s paired 2-tailed t test. Where indicated, analysis of variance was performed to examine differences in response to treatments between groups. A probability value of <0.05 was considered significant.

Results

VEGF Increases G6PD Activity and Localization at Plasma Membrane

Because G6PD is required for VEGF-mediated angiogenesis,14 we hypothesized that VEGF should increase G6PD activity in ECs. Surprisingly, in whole cell lysates treated with VEGF for up to 30 minutes, there was no significant increase. However, it has been shown in cardiomyocytes that G6PD is recruited to the plasma membrane in response to ischemia. Therefore, we measured membrane-localized G6PD after VEGF stimulation (Figure 1A and 1B). There was a 4.5-fold increase in G6PD at the plasma membrane, which peaked at 5 minutes (Figure 1A and 1B). Similar results were also observed by membrane fractionation (data not shown). G6PD activity also increased (~2.1-fold at 5 minutes) in response to VEGF stimulation (Figure 1C). Because many VEGF signal events are mediated by tyrosine phosphorylation, especially by c-Src, we determined the effect of PP2, a specific Src inhibitor on G6PD translocation. As shown in Figure 1D, PP2 completely inhibited G6PD translocation at 5 minutes. These results show that VEGF stimulates G6PD translocation and activation at the plasma membrane in a tyrosine kinase-dependent manner.

VEGF Increases G6PD Tyrosine Phosphorylation, Which Is Inhibited by PP2

Similar to G6PD, Src is activated and recruited to plasma membrane in response to VEGF.20,21 To examine whether G6PD is tyrosine phosphorylated in response to VEGF, we treated ECs with 20 ng/mL VEGF for 0 to 30 minutes. VEGF treatment increased G6PD tyrosine phosphorylation with peak at 5 minutes (Figure 2A). PP2 significantly attenuated G6PD tyrosine phosphorylation suggesting a role for Src in regulating G6PD in response to VEGF (Figure 2B). To facilitate the analysis of critical tyrosine residues in G6PD phosphorylated by VEGF, we transfected ECs with a Flag-tagged G6PD expression construct. VEGF stimulated ty-
rosine phosphorylation of Flag-G6PD in ECs that was completely inhibited by PP2 (Figure 2C and 2D). The small increase in tyrosine phosphorylation of transfected G6PD (1.2-fold) likely reflects the relatively low transfection efficiency of ECs, and the fact that we measured G6PD tyrosine phosphorylation in whole cell extracts rather than plasma membrane sheets. However, the magnitude of inhibition by PP2 was highly significant (Figure 2D).

Figure 1. VEGF increases G6PD activity and localization at plasma membrane. ECs were stimulated with VEGF (20 ng/mL) for the indicated times. A, Plasma membranes were prepared and then fixed and stained for G6PD. The scale bar on each field represents 20 μm. B, The fluorescence intensities in A were quantified with Image J (1.36b, National Institutes of Health). C, Proteins were extracted from plasma membrane sheets of cells stimulated with VEGF (20 ng/mL). G6PD activity was measured in a plate reader. D, Cells were preincubated with 10 μmol/L PP2 for 1 hour and then stimulated with 20 ng/mL VEGF for 5 minutes. After VEGF treatment, plasma membranes were prepared and quantified as in A. Values are mean±SE from 3 independent experiments. *P<0.05 compared to control; **P<0.05 compared to VEGF.

Figure 2. VEGF treatment increases G6PD tyrosine phosphorylation, which is inhibited by PP2. ECs were serum depleted for 24 hours and then treated with VEGF (20 ng/mL). A, After VEGF treatment for the indicated times, protein was harvested and tyrosine phosphorylated proteins were pulled-down by immunoprecipitation using phosphotyrosine antibody 4G10. Tyrosine phosphorylation (pY) of G6PD was detected by Western blot using G6PD antibody. B, ECs after 24 hours of serum depletion were pretreated with 10 μmol/L PP2 for 1 hour before VEGF treatment (20 ng/mL for 5 minutes). Protein was harvested and tyrosine phosphorylated proteins were pulled-down by immunoprecipitation using 4G10. Tyrosine phosphorylation (pY) of G6PD was detected by Western blot using G6PD antibody. C, ECs transfected with Flag-tagged wild-type G6PD (WT) were preincubated with 10 μmol/L PP2 for 1 hour before VEGF treatment (20 ng/mL for 5 minutes). Flag-G6PD was pulled-down from total cell lysates using Flag antibody. Tyrosine phosphorylation of

Flag-G6PD (Flag-G6PD-pY) was assayed by Western blot using 4G10. D, The results of C were quantified by densitometry of autoradiograms. Values are mean±SE from 3 independent experiments. *P<0.05 compared to control. **P<0.05 compared to VEGF-stimulated cells.
Src Phosphorylates G6PD in Response to VEGF

Src is critical for the phosphorylation of several molecules that mediate VEGF signaling.22–24 In ECs, VEGF rapidly induces activation of Src at the plasma membrane.25,26 The above findings suggest that VEGF-activated Src may be responsible for G6PD phosphorylation. To examine whether G6PD is a direct substrate of Src, an in vitro kinase assay was performed using G6PD-GST fusion protein as substrate. For this assay, EC lysates were prepared after VEGF stimulation, and Src was pulled down by immunoprecipitation. The in vitro kinase assay was performed by incubating G6PD-GST with immunoprecipitated Src in the presence of \( ^{32}P\)-ATP. The results show that G6PD is phosphorylated in a time-dependent manner with peak at 5 minutes in response to VEGF (Figure 3). VEGF induces Src autophosphorylation with a similar time course (Figure 3) suggesting that G6PD is a Src substrate.

Basal G6PD Activity Is Dependent on Src

To provide further evidence for the role of Src in G6PD tyrosine phosphorylation, we inhibited Src by 3 different approaches. First, ECs were pretreated with herbimycin A (1.0 \( \mu \text{mol/L} \)) or PP2 (10 \( \mu \text{mol/L} \)), then G6PD activity was measured. Preincubation of ECs with herbimycin A and PP2 inhibited G6PD activity similarly by \( \approx25\% \) (Figure 4A). Second, we used Src-deficient fibroblasts.27 G6PD activity was measured in both SYF cells (fibroblasts deficient in Src, Yes, and Fyn), and in S\(^+\)YF cells (Src reexpressed in SYF cells). G6PD activity was \( \approx22\% \) less in SYF cells (Figure 4B). This decrease in G6PD activity was not attributable to any change in G6PD expression (Figure 4B). These results indicate that Src is required for basal G6PD activity. Third, to examine whether Src kinase activity is required for G6PD activity, we transfected kinase-dead Src (kDa-Src) into ECs. There was a \( \approx30\% \) decrease in G6PD activity in kDa-Src transfected cells compared to vector control (Figure 4C). These results strongly suggest that Src regulates G6PD via tyrosine phosphorylation.

Tyrosine 428 and 507 of G6PD Are Phosphorylated in Response to VEGF

To identify G6PD tyrosine residues likely phosphorylated by Src, we searched for residues conserved through evolution using ExPASy BLAST 2. We found 2 tyrosine residues (Y428 and Y507) that were highly conserved among 8 widely
divergent species (Homo sapiens, Mus musculus, Rattus norvegicus, Cricetulus griseus, Fugu rubripes, Bos indicus, Drosophila melanogaster, and Caenorhabditis elegans). Further motif searches (NetPhos, v.2.0; scansite.mit.edu) suggested that Y428 and Y507 were likely sites for Src kinase phosphorylation.

To characterize specific roles for Y428 and Y507 in G6PD phosphorylation, we made Flag-tagged expression constructs with tyrosines to phenylalanine mutations (G6PD-Y428F, G6PD-Y507F), and double mutation of both tyrosine residues (G6PD-Y428F/Y507F). Then we examined VEGF-mediated G6PD phosphorylation after transient transfection (Figure 5). G6PD-Y428F significantly decreased tyrosine phosphorylation basally (1.00 to 0.19) and in response to VEGF (1.90 to 0.21), whereas G6PD-Y507F and G6PD-Y428F/Y507F almost completely abolished both basal and stimulated tyrosine phosphorylation compared to G6PD-WT (Figure 5). The dramatic inhibition of G6PD phosphorylation by single tyrosine mutations suggests a potentially cooperative mechanism for G6PD activation that requires further investigation. Together, these results indicate that Y428 and Y507 are the sites phosphorylated in response to VEGF, whereas Y507 appears more important for basal phosphorylation.

G6PD Tyrosine Phosphorylation Modulates VEGF-Mediated Akt Phosphorylation and EC Migration

We examined whether tyrosine phosphorylation of G6PD at Y428 and Y507 plays a role in the EC response to VEGF as measured by Akt phosphorylation and EC migration. Transfection of G6PD-WT increased Akt phosphorylation by 1.6-fold after VEGF stimulation. Transfection of G6PD mutants significantly inhibited Akt phosphorylation with rank order of inhibition G6PD-Y428F/Y507F > Y507F > Y428F (Figure 6A and 6B). We further tested the role of G6PD tyrosine phosphorylation in VEGF-induced EC migration. G6PD-WT increased VEGF-induced EC migration by 1.3-fold compared to control in a wound-healing assay, whereas G6PD tyrosine mutants significantly decreased EC migration (Figure 6C). These results indicate that G6PD tyrosine phosphorylation is required for VEGF-induced Akt phosphorylation and cell migration.

Discussion

The major finding of this study is that G6PD activation by VEGF is regulated by Src-mediated tyrosine phosphorylation and translocation to the plasma membrane. Specifically, we showed that Y428 and Y507 of G6PD are required for G6PD activity, Akt phosphorylation and EC migration in response to VEGF. To our knowledge, these findings are the first to demonstrate that G6PD function is regulated by tyrosine phosphorylation in ECs. Our results are supported by the observations that in the Pretsch mice, a murine model of G6PD deficiency, decreased G6PD activity is associated with attenuated EC proliferation, migration, and tube formation in response to VEGF. 14

We found increased G6PD localization at plasma membrane in response to VEGF stimulation (Figure 1A and 1B), which is consistent with the observations that G6PD translocated to the membrane after activation by H2O2 in cardiomyocytes. 9 More interestingly, we found that PP2 treatment dramatically decreased G6PD membrane translocation at 5 minutes of VEGF treatment (Figure 1D) suggesting a causal relationship between Src activity and G6PD membrane translocation. Because G6PD can be phosphorylated by Src (Figure 3), it is likely that Src-mediated tyrosine phosphorylation is a key regulatory mechanism for G6PD membrane translocation.

Although the specific role for membrane localized G6PD requires further studies, our data suggest a redox-related mechanism. We found that tyrosine mutants of G6PD-Y428F/Y507F attenuated G6PD activity (unpublished results). Moreover, G6PD-Y428F/Y507F significantly inhibited VEGF-mediated Akt phosphorylation, and EC migration (Figure 6). As the enzyme that catalyzes NADPH production through pentose phosphate pathway, G6PD is indispensable for the redox homeostasis of the cells. Two key membrane enzymes in ECs that require NADPH are eNOS and NADPH oxidase. Interestingly, it has been demonstrated that both enzymes modulates VEGF signaling and angiogenesis. 14,28,29

Our data suggest that tyrosine phosphorylation of Y428 and Y507 is also important in basal G6PD activity because inhibition of Src by 3 different techniques significantly decreased G6PD activity (Figure 4A and 4C). In addition, basal tyrosine phosphorylation of G6PD was decreased dramatically with mutation of either Y428 or Y507 (Figure 5). These results suggest that assembly of the complex required for G6PD activity requires interactions among proteins that recognize phospho-tyrosines. Interestingly, Src knockout mice (but not Fyn knockouts), exhibit impaired VEGF-induced vascular responses. Further, Src, but not Fyn, interacts with the VEGF receptor, suggesting that Src is the
Here we demonstrated that the same primary tyrosine kinase involved in VEGF regulation of G6PD in ECs.

Because G6PD is a ubiquitously expressed enzyme, the present findings have potentially broader significance. In this regard, it was recently shown that G6PD is phosphorylated at Y507 in small cell lung cancers through a global phosphoproteomics analysis. Here we demonstrated that the same site is regulated by Src-mediated tyrosine phosphorylation in ECs. These results, combined with our current findings, suggest that selective regulation of G6PD tyrosine phosphorylation may represent a novel approach to regulate tumor angiogenesis.

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Disclosures
None.

References


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