Glucose-6-Phosphate Dehydrogenase Overexpression Decreases Endothelial Cell Oxidant Stress and Increases Bioavailable Nitric Oxide

Jane A. Leopold, Ying-Yi Zhang, Anne W. Scribner, Robert C. Stanton, Joseph Loscalzo

Objective—Glucose-6-phosphate dehydrogenase (G6PD), the principal source of NADPH, serves as an antioxidant enzyme to modulate the redox milieu and nitric oxide synthase activity. Deficient G6PD activity is associated with increased endothelial cell oxidant stress and diminished bioavailable nitric oxide (NO). Therefore, we examined whether overexpression of G6PD would decrease reactive oxygen species accumulation and increase bioavailable NO in endothelial cells.

Methods and Results—Adenoviral-mediated gene transfer of G6PD increased G6PD expression, activity, and NADPH levels in bovine aortic endothelial cells (BAECs). BAECs overexpressing G6PD demonstrated a significant reduction in reactive oxygen species accumulation when exposed to hydrogen peroxide, xanthine-xanthine oxidase, or tumor necrosis factor-α compared with BAECs with basal levels of G6PD. BAECs overexpressing G6PD maintained intracellular glutathione stores when exposed to oxidants because of increased activity of glutathione reductase, an effect that was not observed in endothelial cells with normal G6PD activity. Overexpression of G6PD was also associated with enhanced nitric oxide synthase activity, resulting in elevated levels of cGMP, nitrate, and nitrite, and this response was increased after stimulation with bradykinin.

Conclusions—Overexpression of G6PD in vascular endothelial cells decreases reactive oxygen species accumulation in response to exogenous and endogenous oxidant stress and improves levels of bioavailable NO. (Arterioscler Thromb Vasc Biol. 2003;23:411-417.)

Key Words: glucose-6-phosphate dehydrogenase • oxidant stress • endothelium • nitric oxide • nitric oxide synthase

The vascular endothelium modulates cellular oxidant stress by increasing the activity of enzymes with antioxidant properties such as glucose-6-phosphate dehydrogenase (G6PD). G6PD, the first and rate-limiting enzyme of the pentose phosphate pathway, catalyzes the synthesis of riboses for nucleic acid production and is the principal intracellular source of NADPH. NADPH, in turn, is used as a reducing equivalent to maintain reduced glutathione stores (GSH), which are used to scavenge reactive oxygen species (ROS). ROS are buffered intracellularly by converting GSH to its oxidized form (GSSG) in a reaction catalyzed by glutathione peroxidase (GPx). GSSG is recycled to GSH by glutathione reductase (GSSG reductase), which requires NADPH as a cofactor. In this manner, G6PD importantly regulates the intracellular redox milieu.

G6PD has long been recognized as an antioxidant enzyme in erythrocytes, which have no alternative source of NADPH,1 and has recently been shown to mediate ROS accumulation in vascular endothelial and smooth muscle cells.2,3 G6PD-deficient fibroblasts manifest premature senescence in vitro owing to increased local production of ROS,4 and when challenged additionally with hydrogen peroxide (H2O2), demonstrate accelerated cell death.5 Similarly, vascular endothelial cells with deficient G6PD activity exposed to H2O2 generate increased levels of ROS and deplete intracellular GSH stores,2 an effect that is associated with diminished cell viability.5

In the vascular endothelium, G6PD, as the principal source of NADPH, additionally regulates the activity of enzymes that require NADPH as a cofactor, including the endothelial isoform of nitric oxide synthase (eNOS). NADPH binds directly to the reductase domain of eNOS and is required to maintain tetrahydrobiopterin (BH4) stores via de novo synthesis and the dihydrofolate reductase salvage pathway. When these cofactors are not replete, eNOS has been shown to produce superoxide in preference to NO in cell free systems.7,8 We have previously demonstrated the influence of G6PD on eNOS; endothelial cells with deficient G6PD...
activity and reduced NADPH levels demonstrate increased oxidant stress attributable to endogenous eNOS-mediated ROS production concomitant with decreased endothelial-derived bioavailable NO.2

Gene transfer of G6PD has been shown to increase enzyme activity to confer protection against elevated levels of ROS on cells exposed to oxidant stress. HeLa cell clones transduced with G6PD and exposed to exogenous oxidants maintain GSH levels and exhibit decreased susceptibility to oxidant-mediated cell death.9 In addition, overexpression of G6PD has been shown to promote cell proliferation in COS-7 cells10 and protect against H2O2-mediated cell death in PC12 cells.5 In this study, we demonstrate that gene transfer of G6PD in vascular endothelial cells increases intracellular NADPH levels to abrogate endogenous and exogenous oxidant stress and augments levels of endothelial-derived bioavailable NO.

Methods

Cell Culture
Bovine aortic endothelial cells (BAECs) (Cell Systems Co) were grown to confluence in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were passaged twice weekly by harvesting with 0.5% trypsin:EDTA. Experiments were conducted on cells from passages 4 to 12.

Production of Recombinant Adenovirus AdG6PD
An adenoviral G6PD expression vector encoding rat G6PD cDNA, pAdG6PD, was constructed as previously described.11 The sequence of pAdG6PD was confirmed by DNA restriction digestion and after transfection of HEK-293 cells by Western blotting and activity assay of the expressed G6PD protein. To produce the recombinant virus, pAdG6PD was linearized with PstI and transfected into HEK-293 cells using LipofectAMINE (Gibco) as described.11 The virus, AdG6PD, was harvested from the cells by 4 cycles of freezing and thawing in PBS containing 10% (vol/vol) glycerol. Additional viral amplification was performed by several rounds of infection of HEK-293 cells. The titer of the AdG6PD produced was determined by viral plaque assay. For experiments, BAECs were transfected with AdG6PD or an empty viral vector (Ad) for 24 hours in full growth media, washed, and placed in full growth media for an additional 24 hours before assay.

G6PD Activity
G6PD activity was measured as previously described.12 Briefly, enzyme activity was determined in a cell lysate using a plate-reader spectrophotometer (ThermoMax Microplate Reader, Molecular Devices) by measuring the rate of increase of absorbance at 340 nm from the conversion of NADP+ to NADPH by either G6PD or 6-phosphogluconate dehydrogenase (6-PGD). Substrate concentrations used were glucose-6-phosphate (200 μmol/L), 6-phosphogluconate (200 μmol/L), and NADP+ (100 μmol/L).

G6PD and eNOS Immunoblotting
Total cell protein (25 μg cell protein per lane) was size-fractionated electrophoretically using SDS-polyacrylamide gel electrophoresis on a 10% gel and transferred to nitrocellulose membranes blocked with 5% skim milk solution. The membranes were incubated with 1:1000 dilution of a polyclonal rabbit anti-G6PD antibody (Sigma) or with a 1:1000 dilution of a monoclonal mouse anti-eNOS antibody (Transduction Laboratories) and visualized using the ECL detection system (Amersham).

NADPH Determination
NADPH levels were determined spectrophotometrically based on the measurement of the absorbance of the reduced coenzyme at 340 nm (Beckman DU 640B spectrophotometer, Beckman Coulter, Inc), as previously described.11 An initial reading of the cell sample at 340 nm measured total NADPH and NADH levels in the sample. To assay for NADH alone, an aliquot of the cell extract (50 μL) was incubated with glutathione reductase (5.0 IU) for 5 minutes. To start the reaction, 0.005 mol/L glutathione (GSSG) was added and incubated for an additional 5 minutes. NADPH levels were calculated as the difference between total pyridine nucleotides in the sample and NADH levels.

DCF Fluorescence
Cells were grown to confluence in 96-well plates, washed once with PBS (0.9%), and then incubated with 20 μmol/L 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (diacetoxymethyl) ester (Molecular Probes) for 1 hour at 37°C. BAECs were exposed to H2O2, xanthine-xanthine oxidase (X-XO), or tumor necrosis factor (TNF)-α and cellular ROS accumulation was determined in a microplate fluorometer (SpectraMax Gemini, Molecular Devices).13

Cell Death Assays
Apoptotic cell death was determined using the Cell Death Detection ELISAapo25 (Roche) according to the manufacturer’s instructions. Cell necrosis was determined by measuring lactate dehydrogenase levels in the media using a Lactate Dehydrogenase kit (Sigma) according to the manufacturer’s instructions.

Indices of Glutathione Recycling
Intracellular glutathione (GSH) levels were measured as previously described using the Bioxytech GSH-400 enzymatic method (OXIS).2 Total cellular protein was precipitated in 5% metaphosphoric acid and centrifuged, and the supernatant was isolated for measurements.

Cellular glutathione peroxidase (Gpx) and glutathione reductase (GSSG reductase) activities were determined as previously described using the Bioxytech Gpx-340 assay (OXIS) and GR-340 assay (OXIS), respectively.2 In selected experiments, the protocol was modified by omitting the addition of exogenous NADPH. In this manner, the assay relied on endogenous NADPH levels for the reaction.

eNOS Activity
eNOS activity was measured in intact cells without the addition of exogenous cofactors.13 Briefly, cells were incubated for 15 minutes with 0.75 μCi/mL [3H]-L-arginine and selected plates were stimulated with bradykinin (5 μmol/L). The reaction was terminated by the addition of 500 μL ice-cold 1N trichloroacetic acid to each plate. The cells were twice freeze-fractured in liquid nitrogen and scraped from the plates, and ether extraction was performed 3 times with water-saturated ether. The samples were neutralized with 1.5 mL of 25 mmol/L HEPES, pH 8, applied to Dowex AG50Wx-8 (Tris form) columns and eluted with 1 mL 40 mmol/L HEPES buffer, pH 5.5, with 2 mmol/L EDTA and 2 mmol/L EGTA. [3H]-L-citrulline was quantified by liquid scintillation counting. In certain experiments, cells were pretreated with L-NAME (1 mmol/L) for 1 hour to inhibit eNOS activity.

cGMP, Nitrate, and Nitrite Measurements
Confluent BAECs were pretreated with 0.5 mmol/L isobutylmethylxanthine (IBMX) at 37°C for 15 minutes. They were then incubated with bradykinin (5 μmol/L) for 10 minutes in the presence of IBMX. The reaction was stopped by the addition of ice-cold 6% trichloroacetic acid. After 3 freeze-thaw cycles, the cells were scraped from the plate. The supernatant fractions of the cell lysates were extracted with water-saturated ether, dried with nitrogen gas at room temperature, and acetylated. Cyclic GMP levels were quantitated using a cGMP enzyme immunoassay kit (Cayman Chemical Co). Nitrate and nitrite accumulation in the media were assayed using a fluorometric
assay kit (Cayman Chemical Co) according to manufacturer’s instructions.

**Statistical Analysis**

Data are expressed as mean±SEM. Comparison between groups was performed by Student’s paired two-tailed t test. *P*<0.05 was considered significant.

**Results**

**G6PD Overexpression and NADPH Levels**

To examine the effect of G6PD overexpression in BAECs, cells were transfected with increasing AdG6PD virus particle-to-cell ratios (multiplicity of infection [MOI]) or an empty adenoviral vector (Ad). AdG6PD-mediated gene transfer of G6PD resulted in a dose-dependent increase in G6PD protein expression in BAECs that was not observed in endothelial cells transfected with Ad (Figure 1A). Increased G6PD expression in BAECs was associated with a concomitant increase in G6PD activity (Figure 1B). Accordingly, we chose to infect BAECs with the lowest possible concentration of AdG6PD to increase G6PD expression and activity, and all additional experiments were performed with MOI=10 pfu/cell. At this level of G6PD expression, there was a significant increase in NADPH levels (0.05158±0.0031 versus 0.07382±0.0022 mmol/L, *n*=4, *P*<0.012) (Figure 1C).

**G6PD Overexpression and Oxidant Stress**

To determine the influence of G6PD overexpression on protection from exogenous oxidant stress in BAECs, cells overexpressing G6PD were exposed to H$_2$O$_2$ (100 or 500 μmol/L), and intracellular ROS accumulation over time was measured by DCF fluorescence. There was no significant difference in basal levels of ROS production between Ad and AdG6PD transfected endothelial cells; however, after a 30-minute exposure to H$_2$O$_2$ (500 μmol/L), BAECs demonstrated a significant increase in ROS levels compared with BAECs overexpressing G6PD (204.03±18.1 versus 127.2±4.3 fluorescent units, *n*=8, *P*<0.01), and this protective effect was sustained for up to 75 minutes (445.92±38.87 versus 276.07±14.35 fluorescent units, *n*=8, *P*<0.001) (Figure 2A). There was no evidence of H$_2$O$_2$-mediated cell death up to 75 minutes; however, after this time there was a marked change in endothelial cell morphology, an increase in histone-associated DNA fragments (92.69±0.7 versus 1.61±0.1 μU enrichment factor, *n*=4, *P*<0.0001) consistent with apoptosis, and an increase in lactate dehydrogenase levels (283.3±7.5 versus 71.0±5.2 U/mL, *n*=4, *P*<0.0001), an effect that was not seen in BAECs overexpressing G6PD.

To confirm these findings, BAECs were exposed to xanthine (100 μmol/L) and xanthine oxidase (20 μU/mL) for up to 30 minutes and ROS accumulation was determined. BAECs exposed to X-XO demonstrated a time-dependent increase in ROS accumulation that was abrogated in BAECs overexpressing G6PD (Figure 2B).

To demonstrate that G6PD overexpression was similarly protective against an endogenous oxidant stress, BAECs were exposed to TNF-α (100 U/mL), which has been shown to increase ROS production in coronary microvascular endothelial cells. To examine the effect of increased G6PD expression on endogenously generated ROS, BAECs and BAEC overexpressing G6PD were treated with TNF-α (0, 10, 50, or 100 U/mL) for up to 30 minutes and ROS levels were determined. In BAECs exposed to TNF-α, there was a dose- and time-dependent increase in ROS accumulation, which was significantly decreased in AdG6PD-infected BAECs (Figure 2C), thereby demonstrating that G6PD overexpression provided antioxidant benefit against an endogenous, as well as exogenous, oxidant stress.

**Overexpression of G6PD and Glutathione Recycling**

We next measured intracellular GSH levels and GPx and GSSG reductase activities to determine if G6PD overexpression preserved GSH stores by enhanced glutathione recycling. Compared with untreated cells, GSH levels were decreased significantly in Ad-transduced BAECs treated with H$_2$O$_2$ (500 μmol/L) (162.24±9.14 versus 97.63±1.0 μmol/L per mg protein, *n*=4, *P*<0.001), an effect that was not observed after gene transfer of G6PD (133.26±4.1 versus 146.26±5.2 μmol/L per mg protein, *n*=4, *P*=NS) (Figure

![Figure 1. G6PD overexpression increases NADPH levels. A, BAECs were transfected with an adenovirus encoding rat G6PD cDNA (AdG6PD) for 24 hours or an empty viral vector (Ad), and G6PD protein expression was determined by Western blotting. B, Corresponding G6PD activity (units/6 minutes per mg protein) was determined, and data are presented as mean±SEM. *P*<0.001 vs Ad. C, Cellular NADPH levels were measured and reported as mean±SEM. *P*<0.012 vs Ad.](http://atvb.ahajournals.org/Downloaded from June 26, 2017)
Similar results were observed in BAECs and BAECs overexpressing G6PD exposed to X-XO (Figure 3B). In addition, exposure to TNF-α (100 U/mL) decreased GSH levels in Ad-infected BAECs (132.86 ± 8.5 versus 50 ± 2.2 μmol/L per mg protein, n = 4, P < 0.001), an effect that was prevented by overexpression of G6PD in endothelial cells (129.5 ± 10.6 versus 113.0 ± 8.6 μmol/L per mg protein, n = 4, P = NS) (Figure 3C).

In the presence of ROS, GSH is converted to its oxidized form, GSSG, by GPx. To determine if G6PD overexpression maintained GSH levels in the presence of ROS-generating compounds by decreasing the activity of GPx, we next measured enzyme activity. GPx activity was increased in both Ad- and AdG6PD-transduced BAECs after exposure to either H2O2 or TNF-α (Table 1). This suggested that in the presence of ROS, GSH was being converted to GSSG in BAECs overexpressing G6PD.

Once formed, GSSG is recycled to GSH by GSSG reductase in an NADPH-dependent reaction. Therefore, we next examined GSSG reductase activity in a coupled enzyme assay, which requires exogenously added NADPH, to determine if the enzyme was functional and in the absence of exogenously added NADPH to examine the effect of endogenous NADPH levels on enzyme activity. In the presence of exogenous NADPH, GSSG reductase activity was similarly increased in Ad-infected BAECs and BAECs overexpressing G6PD exposed to either H2O2 or TNF-α. Interestingly, in the absence of exogenously added NADPH, BAECs overexpressing G6PD demonstrated increased GSSG reductase activity after exposure to H2O2 (11.7 ± 2.1 versus 26.4 ± 2.8 mU/mL per mg protein, n = 3, P < 0.01) and TNF-α (5.2 ± 1.6 versus 11.9 ± 2.1 mU/mL per mg protein, n = 3, P < 0.05) (Table 1). These observations suggest that one mechanism by which G6PD overexpression maintains GSH stores in the setting of increased ROS production is by enhanced NADPH-dependent glutathione recycling of GSSG to GSH.

**eNOS Activity and G6PD Overexpression**

We have previously shown that decreased G6PD expression impairs the activity of eNOS, which requires NADPH and tetrahydrobiopterin (BH4) (synthesized and recycled in NADPH-dependent reactions) as cofactors. Therefore, we
hypothesized that overexpression of G6PD would enhance eNOS activity. We measured eNOS activity in intact cells in the absence of exogenously added NADPH or BH4. Under basal conditions, overexpression of G6PD significantly increased eNOS activity (17 534 ± 150 versus 54 067 ± 426 cpm [3H]-L-citrulline, n = 3, P < 0.001), and this response was enhanced after stimulation with bradykinin (5 μM/L) for 10 minutes (25 461 ± 253 versus 66 051 ± 184 cpm [3H]-L-citrulline, n = 3, P < 0.001) (Figure 4). This observed increase in eNOS activity was not the result of increased eNOS expression, as determined by Western immunoblotting (data not shown).

To examine the influence of increased eNOS activity on levels of bioavailable NO, we measured cGMP levels in BAECs overexpressing G6PD. At baseline, there was a modest increase in cGMP levels in AdG6PD-transduced BAECs compared with control cells (1.7 ± 0.1 versus 2.8 ± 0.2 pmol cGMP/mg protein, n = 5, P < 0.01), and this effect was enhanced after stimulation with bradykinin (2.9 ± 0.2 versus 4.9 ± 0.1 pmol cGMP/mg protein, n = 5, P < 0.01). As an additional measure of bioavailable NO, we examined nitrate and nitrite levels in BAECs overexpressing G6PD. Under basal conditions, both nitrate (759.24 ± 25.21 versus 1021.18 ± 48.9 pmol/mg protein, n = 4, P < 0.01) and nitrite (41.89 ± 3.1 versus 65.21 ± 5.6 pmol/mg protein, n = 4, P < 0.01) levels were increased in BAECs overexpressing G6PD compared with endothelial cells with ambient levels of G6PD expression, and this response was augmented additionally after cells were stimulated with bradykinin (Table 2).

Discussion

In these studies, we found that gene transfer of G6PD conferred a protective benefit on vascular endothelial cells exposed to either exogenous or endogenous oxidant stress. Overexpression of G6PD was associated with a decrease in intracellular ROS accumulation in endothelial cells treated with H2O2, X-XO, or TNF-α. Furthermore, overexpression of G6PD maintained the intracellular redox milieu by preserving cellular GSH stores in endothelial cells challenged with ROS-generating compounds. Intracellular GSH levels were maintained, in part, because of enhanced glutathione recycling via GSSG reductase in an NADPH-dependent reaction. Because NADPH stores are determined by G6PD activity, this response may therefore be attributed to the increased NADPH levels measured in endothelial cells overexpressing G6PD. Gene transfer of G6PD also enhanced the activity of eNOS, which requires NADPH and BH4 as cofactors, to increase levels of bioavailable NO. Under basal conditions, increased eNOS activity in endothelial cells overexpressing G6PD was associated with elevated levels of cGMP, nitrate, and nitrite, and these indices of bioavailable NO were augmented by stimulation with bradykinin.

G6PD, the first and rate-limiting enzyme of the pentose phosphate pathway, additionally functions as an antioxidant enzyme to maintain intracellular redox balance by serving as the principal intracellular source of NADPH, which is used as a reducing equivalent. Deficient G6PD activity is associated with increased cellular ROS accumulation, resulting in depletion of glutathione stores and enhanced oxidant stress. Therefore, it is not surprising that G6PD overexpression decreases cellular indices of oxidant stress, and there is accumulating evidence to support this hypothesis. In fact, NIH3T3 cells that overexpress G6PD were 2.3 to 3.7 times more resistant to the oxidant tert-butyl hydroperoxide than cells with basal levels of G6PD activity, an effect that was attributed to increased intracellular NADPH and GSH levels. Furthermore, overexpression of G6PD was associated with a reduction in intracellular ROS levels and a decrease in oxidized adducts of lipid peroxidation after exposure to tert-butyl hydroperox-
TABLE 1. Glutathione Peroxidase and Glutathione Reductase Activity

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<th>H₂O₂, 500 μmol/L</th>
<th>TNF-α, 100 U/mL</th>
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<td>GPx activity, mU/mL per mg protein</td>
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<tr>
<td>Ad</td>
<td>369.8±30.2</td>
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<td>AdG6PD</td>
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<td>GSSG reductase activity, mU/mL per mg protein</td>
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<td>With exogenous NADPH</td>
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<tr>
<td>Ad</td>
<td>146.5±18.4</td>
<td>157.2±23.1</td>
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<tr>
<td>AdG6PD</td>
<td>149.7±30.0</td>
<td>173.8±25.7</td>
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<td>Without exogenous NADPH</td>
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<td>Ad</td>
<td>14.5±1.8</td>
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<tr>
<td>AdG6PD</td>
<td>11.7±2.1</td>
<td>26.4±2.8*</td>
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GPx indicates glutathione peroxidase; GSSG reductase, glutathione reductase.
*P<0.01 vs −H₂O₂.
†P<0.05 vs −TNF-α.

Figure 4. G6PD overexpression increases eNOS activity. eNOS activity was determined in BAECs overexpressing G6PD under basal conditions and after stimulation with bradykinin (BK) (5 μmol/L) for 10 minutes. In selected experiments, cells were pre-treated with L-NAME (1 mmol/L) for 60 minutes to inhibit eNOS activity. eNOS activity is measured as [³H]-L-citrulline (cpm), and data are expressed as mean±SEM. *P<0.01 vs no addition; #P<0.001 vs Ad.

TABLE 2. Nitrate and Nitrate Levels

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<tr>
<td>Nitrate, pmol/mg protein</td>
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<tr>
<td>Ad</td>
<td>759.25±25.2</td>
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<tr>
<td>AdG6PD</td>
<td>1021.18±48.9</td>
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<tr>
<td>Nitrite, pmol/mg protein</td>
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</tr>
<tr>
<td>Ad</td>
<td>41.89±3.1</td>
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<tr>
<td>AdG6PD</td>
<td>65.21±5.6</td>
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*P<0.05 vs −BK.
eNOS. We have shown that endothelial cells with deficient G6PD activity have decreased levels of bioavailable NO resulting from an “uncoupling” of eNOS to generate ROS in preference to NO. This uncoupling of eNOS has been shown to occur when cofactors, such as NADPH or BH₄, or substrate, L-arginine, are depleted. This would suggest that overexpression of G6PD, by increasing NADPH levels and thereby BH₄ stores, should increase eNOS activity. Indeed, this is what our studies in aortic endothelial cells overexpressing G6PD demonstrated; increased G6PD expression was associated with enhanced eNOS activity, resulting in augmented cGMP, nitrate, and nitrite levels.

There is a mounting body of evidence to suggest that G6PD, under basal conditions, serves as an antioxidant enzyme in vascular cells in the setting of increased ROS production. In vascular endothelial cells, overexpression of G6PD augmented this antioxidant response and conferred a protective benefit on cells exposed to ROS-generating compounds. Furthermore, overexpression of G6PD was associated with enhanced eNOS-mediated NO generation. Therefore, overexpression of G6PD is one mechanism by which vascular endothelial cells may be protected from increased oxidant stress and maintain levels of bioavailable NO to prevent endothelial dysfunction.

Acknowledgments

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