

# Glucose-6 Phosphate Dehydrogenase Deficiency Decreases the Vascular Response to Angiotensin II

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**Background**—Glucose-6-phosphate dehydrogenase (G6PD) regulates production of the reduced form of NADPH through the pentose phosphate pathway. G6PD may therefore affect superoxide anion production via vascular NADPH oxidase, which is key in mediating the vascular response to angiotensin II (Ang II). We determined the hypertensive and vascular hypertrophic response to Ang II in G6PD-deficient mice.

**Methods and Results**—Ang II (0.7 mg/kg per day) was infused via subcutaneous osmotic pumps for 6 days in male hemizygote G6PD mutant (G6PD<sup>mut</sup>) and wild-type (WT) C3H mice. (1) Compared with WT, G6PD<sup>mut</sup> mouse aorta had 10% to 20% of G6PD activity and 50% less NADPH. (2) Basal systolic blood pressure was not significantly different in G6PD<sup>mut</sup> mice (WT 88±4 mm Hg versus G6PD<sup>mut</sup> 95±4 mm Hg), but Ang II increased blood pressure to a lower level in G6PD<sup>mut</sup> mice (WT 139±4 mm Hg versus G6PD<sup>mut</sup> 123±5 mm Hg;  $P<0.05$ ). (3) Ang II increased aortic medial thickness less in G6PD<sup>mut</sup> mice (WT 71±2 μm versus G6PD<sup>mut</sup> 62±1 μm;  $P<0.01$ ). (4) 3-*o*-Nitrotyrosine staining and dihydroethidium oxidation in the aorta was increased by Ang II less in G6PD<sup>mut</sup> mice. (5) Smooth muscle cells isolated from G6PD<sup>mut</sup> mice showed less Ang II–induced phosphorylation of Akt and p42/44 ERK.

**Conclusions**—G6PD deficiency may reduce vascular superoxide anion production by limiting production of the substrate for NADPH oxidase, thereby inhibiting oxidant-mediated Ang II–induced signaling pathways that contribute to hypertension and smooth muscle hypertrophy. (*Circulation*. 2005;112:257-263.)

**Key Words:** angiotensin ■ aorta ■ free radicals ■ hypertension ■ hypertrophy

Glucose-6-phosphate dehydrogenase (G6PD) regulates production of the reduced form of nicotinamide-adenine-dinucleotide phosphate (NADPH) through the pentose phosphate pathway. Various NADPH-dependent enzymes regulate cellular redox balance, including glutathione reductase, thioredoxin reductase, nitric oxide synthase, and NADPH oxidase. G6PD has been suggested to be an “antioxidant” enzyme because it provides NADPH to maintain glutathione (GSH) in its reduced form. Increased G6PD activity may restore cellular GSH levels after its depletion by oxidative stress.<sup>1,2</sup> In addition, G6PD regulates production of nitric oxide in endothelium and other types of cells.<sup>3–6</sup> On the other hand, superoxide production by the neutrophil NADPH oxidase is decreased in G6PD-deficient granulocytes,<sup>3,4,7</sup> suggesting that vascular NADPH oxidase–dependent superoxide production may be regulated by the concentration of NADPH that is available from the pentose phosphate pathway. In fact, pharmacological inhibition of G6PD was shown to lower NADPH and decrease superoxide production in bovine coronary arteries.<sup>8</sup>

There is increasing evidence that NADPH oxidase is a major source of superoxide anion in vascular tissue<sup>9,10</sup> and

that excess generation of superoxide anion/reactive oxygen species contributes to pathological vascular conditions such as hypertension and atherosclerosis. Angiotensin II (Ang II) is a potent vasoconstrictor and upregulates expression and activity of NADPH oxidase and increases superoxide anion and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) production in vascular smooth muscle cells.<sup>11–15</sup> These reactive oxygen species mediate Ang II–induced signaling events leading to Akt phosphorylation<sup>16–19</sup> and vascular smooth muscle cell hypertrophy.<sup>15</sup> Ang II–induced hypertension and vascular superoxide anion production are markedly blunted in gp91<sup>phox</sup>-deficient<sup>20</sup> and p47<sup>phox</sup>-deficient mice,<sup>21</sup> and aortic medial hypertrophy is also dependent on NADPH oxidase–derived superoxide anion.<sup>20</sup> Thus, the effects of Ang II in the vasculature are tightly linked to NADPH oxidase–dependent reactive oxygen species.

An early clinical report suggested that blood pressure is increased in G6PD-deficient people,<sup>22</sup> but it is not well studied whether risk for cardiovascular disease is affected by G6PD deficiency. We therefore examined the role of G6PD as a source of NADPH in regulating the vascular response to

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Ang II infusion by using G6PD-deficient mutant mice. G6PD-deficient mice demonstrated a less marked hypertensive and vascular hypertrophic response to Ang II. Furthermore, aortic smooth muscle cells isolated from G6PD-deficient mice showed decreased Ang II-induced Akt and ERK phosphorylation, consistent with the lesser medial hypertrophy observed *in vivo*. These findings implicate a unique role of G6PD as a regulator of vascular NADPH levels, NADPH oxidase, and vascular oxidant signaling when the activity of NADPH oxidase is upregulated by Ang II.

## Methods

### Animal Model

The G6PD-deficient mouse model in the C3H strain was bred at our institution from frozen embryos obtained from Medical Research Council (Harwell, UK).<sup>23</sup> This G6PD-deficient mouse line was originally created by Pretsch et al<sup>24</sup> and showed decreased translation of the protein caused by a single mutation in the untranslated region of the X-linked G6PD gene.<sup>25</sup> Mice were genotyped by polymerase chain reaction as previously described.<sup>26</sup> Hemizygous mutant ( $X^{mY}$ , G6PD<sup>mut</sup>) and male wild-type (WT) C3H mice, aged 16 to 20 weeks, were used for Ang II infusion.<sup>27</sup> The mice were anesthetized with inhaled isoflurane, and an incision was made in the midscapular region. Osmotic minipumps (Alzet model 1007D, Alza) containing Ang II (Sigma) dissolved in 0.15 mol/L NaCl and 1 mmol/L acetic acid were implanted, providing a delivery rate of 0.7 mg/kg per day for 6 days. Sham-operated mice were implanted with minipumps containing the solvent alone. Systolic blood pressure was determined by tail-cuff plethysmography (Visitec Systems) before and at the end of Ang II infusion. Ten to 20 repeated values were averaged at each determination. This noninvasive method of measuring blood pressure has been validated in mice and correlates well with intra-arterial measurements made in normotensive and hypertensive mice.<sup>28</sup> These procedures were approved by the Boston University Medical Center Institutional Animal Care and Use Committee.

### Immunohistochemistry for 3-*o*-Nitrotyrosine

Mice were anesthetized with isoflurane and euthanized by exsanguination. The aorta was cleaned of adherent fat, placed in 4% formalin overnight, and dehydrated and embedded in paraffin. Tissue sections (5  $\mu$ m) were obtained from the descending thoracic aorta, 3 mm distal to the left subclavian artery, and processed as previously described.<sup>20</sup> Nonspecific binding was blocked with 10% normal goat serum in PBS (pH 7.4) for 30 minutes before incubation with polyclonal anti-nitrotyrosine antibody (1  $\mu$ g/mL; Upstate Biotechnology) or PBS with 1% BSA overnight at 4°C. Tissue sections were then incubated for 30 minutes at room temperature with biotinylated anti-rabbit IgG (1:800) and developed with the use of a Vectastain ABC kit (Vector). Specificity of anti-3-nitrotyrosine antibody was confirmed by lack of staining with antibody that was preincubated with free 3-nitrotyrosine (10 mmol/L; Sigma) and by elimination of staining after reduction of nitrotyrosine to aminotyrosine with sodium dithionite (100 mmol/L in 100 mmol/L sodium borate for 40 minutes). Semiquantitative analysis of tissue immunoreactivity for nitrotyrosine was done by estimating the degree of staining with the use of an arbitrary grading system from 1 to 4 and was performed by 3 observers who were blinded to the sample identification.<sup>20,27</sup>

### Measurement of Aortic Medial Thickness

Aortic cross sections were stained with hematoxylin and eosin and photographed at a magnification of  $\times 100$ . The microscopic images of these sections were displayed on a computer with the use of Adobe Photoshop software. Four measurements of aortic medial thickness oriented at 90° were made per section with the use of NIH Image software. The increase in medial thickness caused by Ang II is comparable to that in medial area reported previously.<sup>20</sup>

### Detection of Superoxide Anion by Dihydroethidium

The fluorescent dye dihydroethidium (hydroethidine) was used to evaluate superoxide anion generation by a previously described method.<sup>29</sup> Mouse aorta was quickly embedded in OCT compound (Miles) to freeze on dry ice and was cut into 10- $\mu$ m sections. Immediately, dihydroethidium ( $2 \times 10^{-6}$  mol/L; Molecular Probes) was applied to cryostat sections at 37°C for 30 minutes in the dark. Images were obtained by fluorescence microscopy (AXINVERT S100TV) with the use of an excitation wavelength of 488 nm and an emission wavelength of 610 nm.

### Mouse Aortic Smooth Muscle Cells

Vascular smooth muscle cells were isolated from aortas of WT and G6PD<sup>mut</sup> mice by enzymatic digestion. Untreated mice aged 4 to 5 months were used for cell isolation. The medial smooth muscle layer of aortas from 2 mice were denuded of endothelium and incubated in serum-free medium containing collagenase II (218 U/mg, 2 mg/mL), elastase (grade II, 0.75 mg/mL), and trypsin inhibitor (type II, 0.5 mg/mL) at 37°C for 20 minutes. Cells were rinsed and plated in Dulbecco's modified Eagle's medium with nutrient mixture F-12 (DMEM/F12) supplemented with 10% fetal bovine serum, 100  $\mu$ g/mL penicillin G, 100  $\mu$ g/mL streptomycin, and 0.25  $\mu$ g/mL amphotericin B. Cells between passages 6 and 15 were cultured in DMEM/F12 with the 10% fetal bovine serum and made quiescent by replacing the media with DMEM/F12 with 0.1% bovine serum albumin for 48 to 72 hours before the experiments.

### Western Blot

Confluent quiescent vascular smooth muscle cells were stimulated with Ang II (100 nmol/L) as indicated. Cells were washed twice with cold PBS and lysed in modified RIPA buffer (1% NP-40, 0.25% deoxycholic acid, 50 mmol/L Tris, pH 7.4, 1 mmol/L EDTA, 150 mmol/L NaCl, 1 mmol/L NaF, 1 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 10  $\mu$ g/mL leupeptin). Protein concentration was determined with protein assay reagent (Bio-Rad). Samples were separated by SDS-PAGE, transferred onto nitrocellulose membranes, and incubated with primary antibody overnight at 4°C. The proteins were detected with the use of horseradish peroxidase-conjugated secondary antibodies and ECL reagents. Some of the mouse aortas were homogenized in modified RIPA buffer, and protein expression for G6PD and other molecules was studied as well. G6PD antibody (polyclonal) was obtained from Bethyl Laboratories.  $\alpha$ -Actin antibody (monoclonal) was from Sigma, and  $\beta$ -actin antibody (monoclonal) was from Abcam. Manganese superoxide dismutase (MnSOD) antibody was from Stressgen. All other antibodies were from Cell Signaling Technology.

### Determination of G6PD Activity

Tissue or cells were homogenized in 20 mmol/L Tris buffer with 0.35 mol/L sucrose and centrifuged at 12 000g for 5 minutes. Supernatant was analyzed for protein concentration, and enzymatic activity of G6PD was assayed according to the method described elsewhere.<sup>30</sup> Briefly, samples were incubated in buffer (50 mmol/L Tris, 1 mmol/L  $MgCl_2$ , pH 8.1) containing substrates 6-phosphogluconic acid and NADP with or without D-glucose 6-phosphate (final concentration: 250  $\mu$ mol/L each). The increase in absorbance at 340 nm ( $V_{max}$ ) was recorded on a plate reader, Spectra MAX 340, equipped with analytical software SoftMax Pro3.0. The difference between readings with and without D-glucose 6-phosphate was taken as G6PD activity.

### Determination of NADPH in Aortic Homogenate

The content of NADPH in G6PD<sup>mut</sup> mouse aorta was determined by measuring the absorbance at 340 nm with or without glutathione reductase as described elsewhere.<sup>6,31</sup> Similarly, the level of NADPH was measured in mouse smooth muscle cells after extraction in buffer (0.1 mole/L Tris, pH 8.0, 0.01 mol/L EDTA, 0.05% [vol/vol] Triton X-100).

### Effect of G6PD Deficiency on Blood Pressure

Experimental Condition	WT	G6PD <sup>mut</sup> Hemizygous	<i>P</i>
Before Ang II infusion	88.1 ± 4.4 (n=13)	94.9 ± 4.1 (n=19)	0.26
After Ang II infusion	139.7 ± 4.7 (n=13)	123.5 ± 5.2 (n=19)	<0.05
Change in pressure with Ang II infusion	51.6 ± 3.0 (n=13)	35.3 ± 5.6 (n=19)	<0.05

Ang II (0.7 mg/kg per day) was infused through subcutaneous osmotic pumps for 6 days. Blood pressure was measured by tail cuff. Data are expressed as mean ± SE (mm Hg). The pressor response to Ang II was significantly less in G6PD<sup>mut</sup> mice.

### Data Analysis

Data are expressed as mean ± SE. Statistical comparisons were performed by ANOVA and Student *t* test. Significance was accepted when *P* was <0.05.

## Results

### Blood Pressure and Body Weight

Although baseline blood pressure tended to be higher in G6PD<sup>mut</sup> mice compared with age-matched C3H WT mice, it was not significantly different (Table). After 6 days of Ang II infusion, blood pressure was significantly increased in both mouse strains, but the pressure increase was significantly less in G6PD<sup>mut</sup> mice, and the final blood pressure was significantly lower in G6PD<sup>mut</sup> mice (Table). Body weight was not significantly different between WT and G6PD<sup>mut</sup> mice before or after surgery (data not shown).

### G6PD Activity and NADPH Content in Mouse Aorta

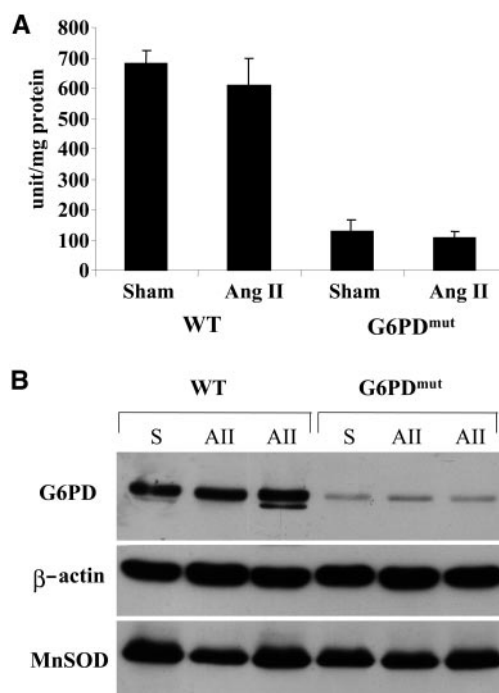
The G6PD enzyme activity in G6PD<sup>mut</sup> mouse aorta was 10% to 20% of that measured in WT mouse aorta and was not altered after Ang II infusion (Figure 1a). Protein expression of G6PD was obviously lower in G6PD<sup>mut</sup> mouse aorta and was not altered after Ang II infusion (Figure 1b). The content of NADPH in G6PD<sup>mut</sup> mouse aorta was ≈50% of that in WT mice (WT 123 ± 7 nmol/mg protein, G6PD<sup>mut</sup> 63 ± 1 nmol/mg protein; n=6; *P*<0.01).

### Aortic Medial Thickness

The cross-sectional medial thickness was not different between WT mice and G6PD<sup>mut</sup> mice after sham infusion (WT 55 ± 2.6 μm, G6PD<sup>mut</sup> 54 ± 2.7 μm). Ang II infusion significantly increased aortic medial thickness to 71 ± 2.0 μm in WT mice and to 62 ± 1.5 μm in G6PD<sup>mut</sup> mice. Compared with WT, the hypertrophic response to Ang II was significantly less in G6PD<sup>mut</sup> mouse aorta (*P*<0.01; Figure 2).

### Immunohistochemistry for 3-*o*-Nitrotyrosine

Ang II infusion increased 3-*o*-nitrotyrosine in mouse aorta,<sup>20,27</sup> and this increase was attenuated in mice deficient in gp91<sup>phox</sup> or in mice that overexpress human superoxide dismutase.<sup>27</sup> Therefore, formation of 3-*o*-nitrotyrosine in proteins was examined as a marker of superoxide generation and its reaction product with nitric oxide, peroxynitrite. In Ang II-infused WT mice, staining for 3-nitrotyrosine on aortic cross sections showed increased staining in the endothelium and the adventitia and to a lesser extent in the media, as shown previously.<sup>20,27</sup> In contrast, the staining in Ang II-infused G6PD<sup>mut</sup> mouse aorta was

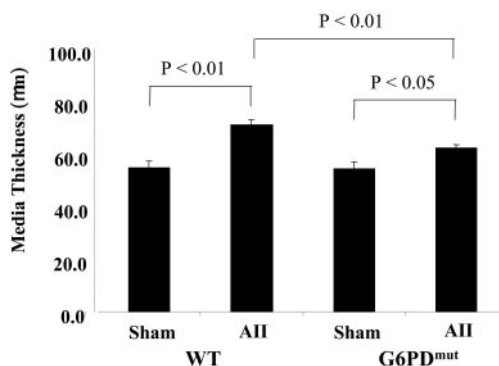


**Figure 1.** G6PD activity and protein expression in Ang II-infused mouse aorta. a, G6PD activity. G6PD was assayed as described in Methods. Ang II infusion (n=5 to 7) did not change G6PD activity in aorta compared with sham (n=4) in either WT or G6PD<sup>mut</sup> mice. b, G6PD expression. G6PD expression did not show any apparent change during Ang II infusion in either type of mice. The antioxidant enzyme MnSOD was expressed comparably in G6PD<sup>mut</sup> mouse and WT mouse aortas.

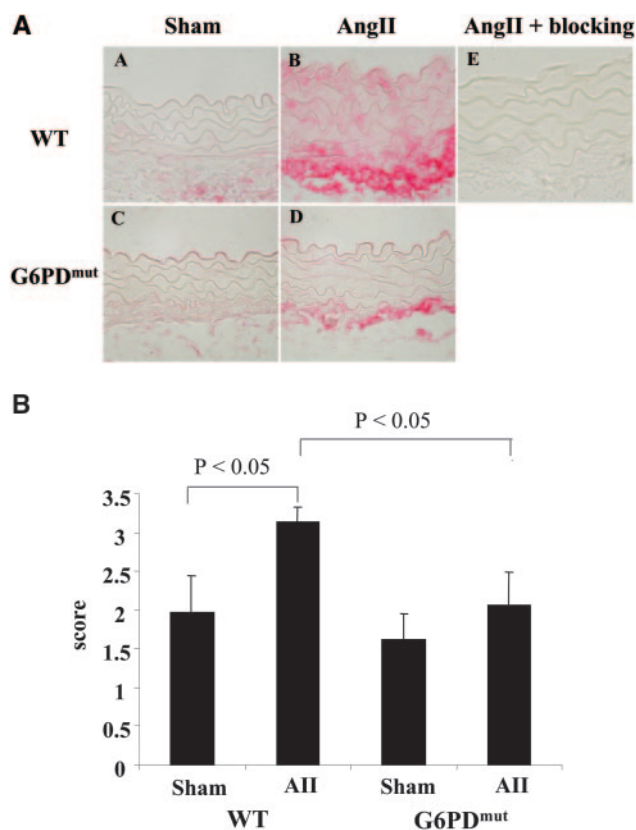
obviously less (Figure 3a). Semiquantitative analysis showed that Ang II infusion significantly increased nitrotyrosine staining compared with saline infusion in WT mice but not in G6PD<sup>mut</sup> mice (Figure 3b).

### Detection of Superoxide Anion by Dihydroethidine in Mouse Aorta

To further assess whether a lower NADPH level results in lower superoxide generation by NADPH oxidase, oxidation



**Figure 2.** Effect of G6PD deficiency on aortic medial thickness. Aortic medial thickness after Ang II or solvent (sham) infusion was measured as described in Methods. Compared with sham (n=4), a significant increase in medial thickness was observed in both WT and G6PD<sup>mut</sup> mice after Ang II (n=7 to 8), but a significantly decreased response was observed in G6PD<sup>mut</sup> mice compared with WT (*P*<0.01).



**Figure 3.** a, Nitrotyrosine staining of G6PD<sup>mut</sup> and WT mouse aorta. Immunohistochemical staining of aortic cross sections for 3-nitrotyrosine in WT and G6PD<sup>mut</sup> mouse infused with Ang II is shown. Photographs were taken from aortas of sham-operated WT mouse (A), Ang II-infused WT mouse (B), sham-operated G6PD<sup>mut</sup> mouse (C), Ang II-infused G6PD<sup>mut</sup> mouse (D), and Ang II-infused WT mouse after preincubation of the antibody with 3-nitrotyrosine to block its immunoreactivity (E). Representative photos are shown. Note that medial thickness is less in the G6PD<sup>mut</sup> mouse aorta. b, Semiquantitative analysis of nitrotyrosine staining of mouse aorta. Nitrotyrosine staining of aortic cross sections was scored by 3 blinded-observers using an arbitrary grade from 1 to 4. No difference was observed between sham-infused G6PD<sup>mut</sup> and WT mouse aorta (n=4). Ang II significantly increased staining in WT mouse but not in G6PD<sup>mut</sup> mouse aorta (n=7 to 8).

of dihydroethidium was examined in mouse aorta. Ang II-infused mouse aorta showed a higher fluorescent signal localized primarily in medial smooth muscle compared with saline-infused mouse aorta (Figure 4a). In addition, Ang II-infused WT mouse aorta showed a visibly greater signal than Ang II-infused G6PD<sup>mut</sup> mouse aorta (Figure 4b).

### Ang II Signaling in Aortic Smooth Muscle Cells

Smooth muscle cells isolated from G6PD<sup>mut</sup> mouse aorta had growth rates similar to those of cells from WT mice.  $\alpha$ -Actin was confirmed by immunoblot as a marker of smooth muscle cells and was expressed similarly and consistently in cells from WT and G6PD<sup>mut</sup> mice during passage. After 10 passages, the G6PD activity of cells from G6PD<sup>mut</sup> mice was <10% of that in cells from WT mice (data not shown). The content of NADPH in cultured cells from G6PD<sup>mut</sup> mice was significantly decreased compared with cells from WT mice (WT 135 $\pm$ 15 nmol/mg protein, G6PD<sup>mut</sup> 88 $\pm$ 4 nmol/mg

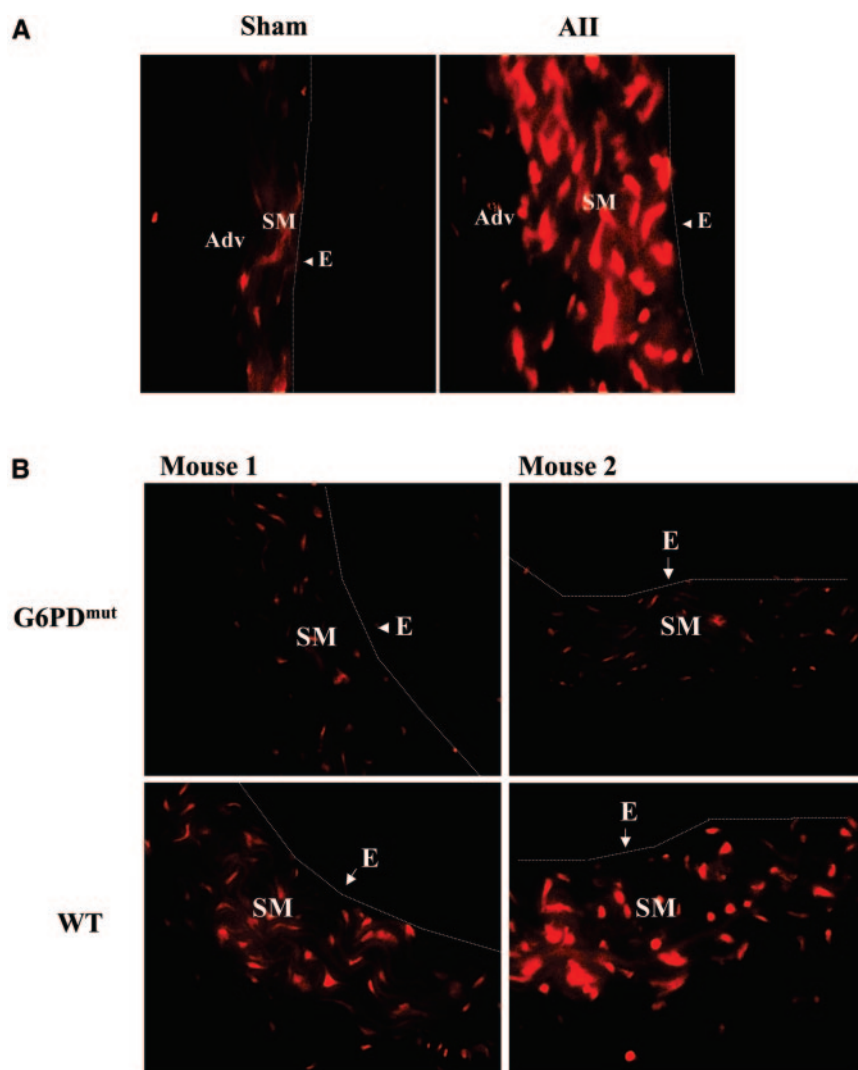
protein; n=3;  $P<0.05$ ). When quiescent cells were stimulated with Ang II, phosphorylation of Akt was lower in cells from G6PD<sup>mut</sup> mice than in cells from WT mice. Additionally, phosphorylation of p42/44 ERK responded less to Ang II in cells from G6PD<sup>mut</sup> (Figure 5).

### Discussion

Our results indicate that vascular responses to Ang II are blunted in G6PD-deficient mice, which is consistent with less NADPH being available for superoxide generation by NADPH oxidase. G6PD<sup>mut</sup> mice showed a smaller pressor response and less medial hypertrophy, superoxide anion, and nitrotyrosine in the aorta. Cultured smooth muscle cells isolated from G6PD<sup>mut</sup> mice also demonstrated attenuated responses to Ang II, confirming that decreased G6PD activity is associated with a decreased response to Ang II at a cellular level.

G6PD is the rate-limiting enzyme in the pentose phosphate pathway that generates 2 molecules of NADPH from NADP<sup>+</sup> through the oxidation of glucose-6-phosphate. Because the pentose phosphate pathway is a main source of cytosolic NADPH, the activity of G6PD may influence various NADPH-dependent reactions. For instance, GSH reductase requires NADPH to maintain GSH in its reduced form. Acute oxidative stress associated with depletion of GSH was shown to increase G6PD expression and to restore GSH levels.<sup>1</sup> In response to acute ischemia/reperfusion, the G6PD-deficient mice used in the present study demonstrated greater cardiac dysfunction, associated with lower GSH levels.<sup>26</sup> Thus, an inadequate supply of NADPH may be a disadvantage in G6PD deficiency by preventing recovery of acutely lowered GSH levels. In addition, G6PD activity may regulate nitric oxide synthase, which also requires NADPH as a cofactor.<sup>3,5,6</sup> NADPH also is required for the activity of NADPH oxidase, and decreased production of superoxide anion and hydrogen peroxide has been reported in G6PD-deficient granulocytes<sup>3,7</sup> and in coronary arteries after pharmacological inhibition of G6PD.<sup>8</sup> The Michaelis constant ( $K_m$ ) for NADPH, 0.04 mmol/L, is 100-fold higher for NADPH oxidase<sup>32,33</sup> than for nitric oxide synthase<sup>34,35</sup> and 5-fold higher than for glutathione reductase.<sup>36</sup> Therefore, NADPH oxidase requires higher concentrations of NADPH to be active and thus may be more susceptible to a decreased supply of the substrate compared with these other NADPH-dependent enzymes. This may be particularly so when NADPH oxidase is activated, as in response to Ang II. In cases in which oxidative stress is mediated by NADPH oxidase, particularly in more chronic conditions, G6PD deficiency would be expected to decrease superoxide anion generation.

It is possible that lower NADPH levels produce less endothelium-derived nitric oxide in G6PD<sup>mut</sup> mice. Consistent with this assumption, basal blood pressure tended to be higher in G6PD<sup>mut</sup> mice. Lower nitric oxide levels, not only lower superoxide, might also explain the decreased nitrotyrosine found in the aorta of G6PD<sup>mut</sup> mice. In preliminary studies, we examined the effect of inhibiting nitric oxide synthase with *N*<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME) on blood pressure in G6PD<sup>mut</sup> mice. L-NAME (1 mg/mL added to the drinking water for 3 weeks) increased blood pressure to



**Figure 4.** Dihydroethidium staining of mouse aorta. After 6-day infusion of Ang II or solvent, mouse aorta was frozen and cross sectioned, followed by dihydroethidium staining as described in Methods. a, Compared with aorta from sham-treated WT mouse (left), aorta from an Ang II-infused WT mouse (right) shows increased staining of the media. b, Aorta from 2 Ang II-infused G6PD<sup>mut</sup> mice (top) show decreased staining compared with 2 aortas from Ang II-infused WT mice (bottom). Adv indicates adventitia; SM, smooth muscle layer; and E, endothelium.

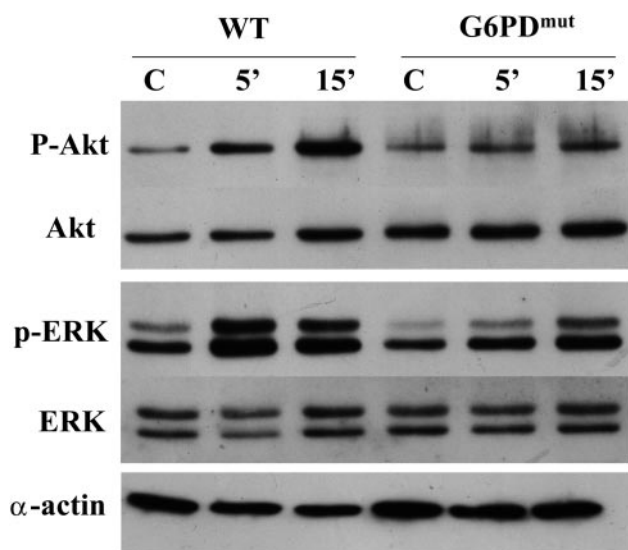
similar levels in WT and G6PD<sup>mut</sup> mice, suggesting that baseline nitric oxide production was not very different. When Ang II was infused together with L-NAME, 3 of 4 WT mice died during the 6 days infusion, whereas 3 of 4 G6PD<sup>mut</sup> mice survived. This suggests that the response to Ang II was blunted in G6PD<sup>mut</sup> mice even after inhibition of nitric oxide synthesis.

Nitrotyrosine staining, a footprint of peroxynitrite derived from superoxide and nitric oxide, was enhanced in adventitia and endothelium after Ang II infusion in WT mouse aorta, consistent with previous observations<sup>20,27</sup> and attributable to superoxide generation from adventitial fibroblasts.<sup>37</sup> However, dihydroethidine indicated intense superoxide production in the smooth muscle layer after Ang II infusion, in agreement with others.<sup>21</sup> Dihydroethidine is accumulated from the reaction of superoxide during a brief *in vitro* incubation, whereas nitrotyrosine accumulates over time *in vivo*. We speculate that the reaction between superoxide and nitric oxide occurs *in vivo* preferentially in adventitia and endothelium. G6PD<sup>mut</sup> mice may demonstrate less nitrotyrosine because of lower superoxide and nitric oxide levels, yielding less peroxynitrite.

One controversial issue relates to substrate specificity of vascular NADPH oxidase. Studies of the oxidase in homogenates of cultured rat aortic smooth muscle cells indicate that both NADH and NADPH can serve as substrates for producing superoxide,<sup>11,38,39</sup> whereas the phagocyte oxidase exclusively consumes NADPH.<sup>10</sup> However, NADPH was by far the preferred substrate in membrane fractions of rabbit aorta.<sup>40</sup> Our results are consistent with NADPH being the more important substrate for vascular NAD(P)H oxidase *in vivo* and are consistent with a recent publication showing that G6PD-dependent NADPH regulates basal superoxide generation in isolated arteries.<sup>41</sup>

The level of NADPH in the aorta or smooth muscle cells in culture was decreased in G6PD<sup>mut</sup> mice to  $\approx 50\%$  of WT mice, even though G6PD activity was 10% that of WT mice. This is likely due to other NADPH-producing enzymes such as malate dehydrogenase and isocitrate dehydrogenase. We speculate that the decrease in NADPH levels manifests itself only when NADPH consumption is unusually increased, such as with induction of an enzyme like NADPH oxidase that has a relatively high  $K_m$  for the substrate.

Earlier studies demonstrated that Ang II activated vascular NADPH oxidase in the plasma membrane to generate super-



**Figure 5.** Ang II-induced Akt and ERK phosphorylation in cultured mouse aortic smooth muscle cells. Quiescent aortic smooth muscle cells isolated from WT and G6PD<sup>mut</sup> mice were stimulated with Ang II (100 nmol/L) for the indicated times. Cellular protein was extracted, and expression of each protein (phospho-Akt, Akt, phospho-p42/44 ERK, p42/44 ERK,  $\alpha$ -actin) was evaluated by Western blot. Representative blots from 4 similar experiments are shown.

oxide/reactive oxygen species.<sup>11,42</sup> Superoxide anion and its metabolite, hydrogen peroxide, are essential regulators of signaling induced by Ang II, leading to phosphorylation of Akt, increased protein synthesis, and medial hypertrophy.<sup>15,16</sup> Because medial hypertrophy was decreased in G6PD<sup>mut</sup> mice, we examined Ang II-mediated signaling in cultured mouse aortic smooth muscle cells. Ang II-induced Akt and p42/44 ERK phosphorylation was relatively decreased in cells from G6PD<sup>mut</sup> mice compared with WT cells. In rat aortic smooth muscle cells, Ang II-induced Akt phosphorylation and protein synthesis are redox sensitive,<sup>16,19</sup> but ERK phosphorylation may not be.<sup>17,19</sup> However, there are also some reports that phosphorylation of ERK in response to Ang II is redox sensitive.<sup>43,44</sup> Compared with rat aortic smooth muscle cells in culture, we found that it is more difficult to consistently decrease basal phosphorylation of ERK in quiescent mouse aortic smooth muscle cells. This difference in cultured cells could explain the difference in the response of ERK. The decreased response in Akt of cells from G6PD<sup>mut</sup> mice provides a molecular mechanism whereby lower levels of NADPH and less oxidant generation can result in decreased medial hypertrophy.

The response to Ang II is not only important in hypertension but also plays a role as a mediator in atherosclerosis<sup>45,46</sup> and angiogenesis.<sup>47</sup> Insufficient substrate for NADPH oxidase might therefore influence cellular responses in various pathologies in vivo. In fact, we found that G6PD deficiency also results in decreased aortic superoxide and atherosclerotic lesions in apolipoprotein E-deficient mice.<sup>48</sup>

G6PD deficiency is the most common enzymopathy in the world and is particularly prevalent in Mediterranean and African countries. G6PD deficiency is known to manifest itself in chemically induced hemolytic anemia but also is

associated with higher resistance to and survival from malaria.<sup>49</sup> It is not well studied whether cardiovascular risk is affected in people with G6PD deficiency. Although an early report suggested a higher incidence of hypertension,<sup>22</sup> a recent cohort study suggested lower mortality from cardiovascular disease in people with G6PD deficiency.<sup>50</sup> Although human cardiovascular diseases stem from multiple genetic and environmental factors, a decreased role of NADPH oxidase may provide a beneficial effect among people with G6PD deficiency. In conclusion, our data suggest that G6PD deficiency may provide an advantage by protecting against Ang II-dependent hypertension by decreasing the contribution of NADPH oxidase-derived superoxide anion to the pressor and hypertrophic vascular response.

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