Arginine therapy of transgenic-knockout sickle mice improves microvascular function by reducing non-nitric oxide vasodilators, hemolysis, and oxidative stress

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Kaul DK, Zhang X, Dasgupta T, Fabry ME. Arginine therapy of transgenic-knockout sickle mice improves microvascular function by reducing non-nitric oxide vasodilators, hemolysis, and oxidative stress. Am J Physiol Heart Circ Physiol 295: H39–H47, 2008. First published May 2, 2008; doi:10.1152/ajpheart.00162.2008.—In sickle cell disease, nitric oxide (NO) depletion by cell-free plasma hemoglobin and/or oxygen radicals is associated with arginine deficiency, impaired NO bioavailability, and chronic oxidative stress. In transgenic-knockout sickle (BERK) mice that express exclusively human α- and β-globins, reduced NO bioavailability is associated with induction of non-NO vasodilator enzyme, cyclooxygenase (COX)-2, and impaired NO-mediated vascular reactivity. We hypothesized that enhanced NO bioavailability in sickle mice will abate activity of non-NO vasodilators, improve vascular reactivity, decrease hemolysis, and reduce oxidative stress. Arginine treatment of BERK mice (5% arginine in mouse chow for 15 days) significantly reduced expression of non-NO vasodilators COX-2 and heme oxygenase-1. The decreased COX-2 expression resulted in reduced prostaglandin E2 (PGE2) levels. The reduced expression of non-NO vasodilators was associated with significantly decreased arteriolar dilation and markedly improved NO-mediated vascular reactivity. Arginine markedly decreased hemolysis and oxidative stress and enhanced NO bioavailability. Importantly, arteriolar diameter response to a NO donor (sodium nitroprusside) was strongly correlated with hemolytic rate (and nitrotyrosine formation), suggesting that the improved microvascular function was a response to reduced hemolysis. These results provide a strong rationale for therapeutic use of arginine in sickle cell disease and other hemolytic diseases.

sickle cell disease; microvascular regulation; vasoreactivity

ACCUMULATING EVIDENCE SHOWS reduced bioavailability of nitric oxide (NO) in human sickle cell disease (SCD) and transgenic-knockout sickle (BERK) mice (7, 10, 19). SCD is characterized by hemolytic anemia and reperfusion injury (excessive oxidant generation), both of which contribute to NO consumption and/or inactivation (1, 16, 31, 36).

NO regulates vascular tone, quenches reactive oxygen species, and has anti-inflammatory effects (5, 23, 26, 31, 33). Under in vivo conditions, catalytic action of NO synthase (NOS) on the substrates arginine and oxygen results in the generation of NO and citrulline. In both human sickle cell anemia (SCA) and sickle mouse models, a preponderance of recent studies have shown depleted levels of l-arginine and NO metabolites (NOx) (8, 29, 30, 39). In SCD, arginine depletion may occur as a result of initial increase in NOS activity and excessive NO generation in response to NO consumption and/or inactivation by cell-free plasma heme and/or oxygen radicals (16, 19, 36). Additionally, conversion of arginine to ornithine by the enzyme arginase may further exacerbate arginine deficiency (16, 28). Thus the combination of hemolysis, reperfusion injury, and reduced NO bioavailability will have adverse consequences on the clinical course of SCD.

Impaired NO bioavailability in human SCD is indicated by diminished responses to sodium nitroprusside (SNP), NOS inhibitors (11, 36), and by reduced flow-mediated vasodilation (7, 11). Sickle mouse models also show attenuated responses to NO-mediated vasoactive stimuli (16, 19, 20, 32). Under the conditions of chronic hypoxia and hemolysis, induction of non-NO vasodilators, such as prostaglandins and heme oxygenase (HO)-1, may compensate for reduced NO bioavailability in SCD (11, 31). Our laboratory has previously demonstrated that reduced NO bioavailability in transgenic-knockout sickle (BERK) mice that express exclusively human α- and β-globins is associated with COX-2 induction in microvascular endothelium (19), suggesting that COX-2 activity is a likely source of elevated PGE2 levels in sickle patients (12). Furthermore, induction of COX-2 in BERK mice is associated with dilatation of resistance vessels (arterioles), hyperperfusion, and hypotension (19). Likewise, human SCD is characterized by hyperperfusion and reduced peripheral resistance (3, 25). Thus activation of non-NO vasodilator mechanisms probably compensates for NO deficiency and helps maintain optimal oxygen delivery in the face of chronic anemia.

As NO has anti-inflammatory properties, reduced NO bioactivity in sickle patients and transgenic sickle mice will contribute to the reported inflammatory effects and increased oxidant generation (1, 14). NO deficiency in BERK mice results in markedly greater oxidative stress, as evidenced by excessive oxygen radical generation, lipid peroxidation, and reduced activity of antioxidants (1, 5). Oxygen radicals can inactivate NO (1, 16) and are implicated in hemolysis (13, 42). Oxidative stress is significantly ameliorated by arginine supplementation (5), and treatment with NO donors can alleviate reperfusion injury (15), in accordance with anti-inflammatory effect of NO (22). In contrast, NOS inhibitor Nω-nitro-L-arginine methyl ester (L-NAME) results in marked inflammatory response (5, 23).

We hypothesize that replenishment of NO in sickle mice will abate activity of non-NO vasodilators, improve vascular tone and responses, decrease hemolysis, and enhance NO bioavailability. Here, we show that arginine supplementation of BERK...
mice decreases expression of non-NO vasodilators COX-2 and HO-1, improves microvascular function, and reduces hemolysis and oxidative stress. Importantly, we show that the improved microvascular function following arginine supplementation is a response to reduced hemolysis and oxidative stress.

MATERIALS AND METHODS

Transgenic Mice

Transgenic-knockout sickle (BERK) mice, expressing exclusively human α- and βββ-globins and generated by Paszty et al. (35), were bred at Albert Einstein College of Medicine, as described (35, 40). C57BL/6J and BERK hemizygous mice (BERK-Hemi mice) were used as controls. BERK-Hemi mice are homozygous for the α-knockout, hemizygous for the β-knockout, and hemizygous for the BERK transgene and express 100% αβ, 15% ββ, and 85% mouse β-globin. Globin composition was determined by HPLC (9). BERK mice were backcrossed for eight or more generations with C57BL/6J mice. BERK-Hemi mice are homozygous for the BERK transgene and express 100% αβ; MCHC, mean corpuscular hemoglobin concentration; Hemi, hemizygous. *P < 0.001 vs. untreated BERK mice; †P < 0.04 vs. untreated BERK-Hemi mice; ‡P < 0.001 vs. untreated BERK mice; §P < 0.001 vs. untreated BERK-Hemi mice.

Globin composition was determined by HPLC (9). BERK mice were bred at Albert Einstein College of Medicine, as described (35, 40). BERK-Hemi mice are homozygous for the BERK transgene and express 100% αβ; and water ad libitum. Sickle mice were maintained on “sickle chow” developed by Paszty et al. (35) without added arginine (Purina Mills, St. Louis, MO). All experimental protocols were approved by the institutional animal studies committee.

Arginine Supplementation

C57BL/6J controls (n = 40), BERK (n = 41), and BERK-Hemi (n = 53) mice, weighing ~25-30 g (4–6 mo old), were supplemented with 5% arginine in mouse chow (Harlan Teklad, Madison, WI) for 15 days. The untreated controls received mouse chow without arginine.

Western Blots

Cremaster tissue lysates were prepared as described (19), and protein concentration was determined at 320 nm. Western blots were performed using 7.5% SDS-PAGE [endothelial NOS (eNOS)], 10% SDS-PAGE (COX-1, COX-2, and HO-1), and a 4–20% linear gradient SDS-PAGE (nitrotyrosine). The membranes were incubated in blocking buffer with antibodies to eNOS (Transduction Laboratory, Lexington, KY; 1:2,000); COX-1 (Santa Cruz Biotechnology; 1:500), COX-2 (Cayman Chemical, Ann Arbor, MI; 1:1,000); HO-1 (Stressgen, Victoria, BC, Canada; 1:3,000); and nitrotyrosine (clone I1A5; Upstate Biotechnology, Lake Placid, NY; 1:800). Incubation with a primary antibody was followed by horseradish peroxidase-conjugated antibody, as described (19). To ascertain equal loading of samples, the stripped membranes were reincubated with goat anti-actin antibody, followed by incubation with anti-goat IgG-horseradish peroxidase secondary antibody. The specific proteins were detected by enhanced chemiluminescence (Duponl, Wilmington, DE). The protein bands on the developed film were quantified by ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Microcirculatory Studies

Mice were anesthetized intraperitoneally with 10% urethane and 2% α-chloralose in saline (5 ml/kg), tracheostomized, the right jugular vein was cannulated for infusion of vasactive substances, and the left carotid artery was cannulated for monitoring arterial pressure. In vivo microscopic observations were made in the transilluminated open cremaster muscle preparation, prepared according to the method of Baer (2), with modifications as described (17).

Vessel luminal diameter was measured online in A2, A3, and A4 arterioles using an image-shearing device (model 907, Instruments for Physiology and Medicine, San Diego, CA). Wall shear rates and volumetric flow (Q) were calculated from vessel diameters and mean flow velocity (centerline red cell velocity/1.6), as described (19).

Arteriolar responses to topical application of acetylcholine (ACH; Sigma, St. Louis, MO) (10^-6 M) and SNP (Sigma, St. Louis, MO) (10^-6 M) were determined in controls and BERK mice, as described (19). In separate experiments, mean arterial pressure (MAP) response to the infusion of L-NAME (Sigma/Aldrich) was evaluated in controls and BERK mice. L-NAME (20 mg/kg) was infused intravenously over 10 min, and MAP was measured after 20 min.

Measurement of Hemolysis and Hematological Parameters

For plasma-free hemoglobin, blood samples were drawn from the abdominal aorta, and determinations were made using a tetramethylbenzidine-based assay kit (Catachem, Bridgeport, CT) that measures plasma-free hemoglobin and other heme-containing proteins present in plasma. Blood samples obtained from tail were analyzed for the mean corpuscular hemoglobin (MCH) and percent reticulocytes using the Bayer Advia 120 (Tarrytown, NY). Hct was measured using a microhematocrit centrifuge (MicroHematocrit, Damon/IEF Division, Needham Heights, MA).

Table 1. Hematological parameters in C57BL, BERK-Hemi, and BERK mice: effect of arginine

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Plasma Hemoglobin, μmol heme</th>
<th>Hct, %</th>
<th>Reticulocytes, %</th>
<th>MCHC, g/dl</th>
<th>MCH, pg/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL untreated</td>
<td>2.7±0.3</td>
<td>48.1±0.8</td>
<td>1.7±0.3</td>
<td>32.5±0.2</td>
<td>14.7±0.2</td>
</tr>
<tr>
<td>C57BL + 5% arginine</td>
<td>2.2±0.2</td>
<td>46.3±0.8</td>
<td>2.6±0.4</td>
<td>32.5±0.3</td>
<td>14.5±0.3</td>
</tr>
<tr>
<td>BERK-Hemi untreated</td>
<td>2.7±0.6</td>
<td>36.1±1.2 *</td>
<td>7.0±0.4 *</td>
<td>30.1±0.2</td>
<td>9.3±0.1 *</td>
</tr>
<tr>
<td>BERK-Hemi + 5% arginine</td>
<td>3.3±0.6</td>
<td>40.2±1.3 †</td>
<td>5.6±0.3 †</td>
<td>31.4±1.0</td>
<td>9.8±0.5</td>
</tr>
<tr>
<td>BERK untreated</td>
<td>7.7±0.5$§</td>
<td>17.1±2.0$§</td>
<td>33.4±2.4$§</td>
<td>20.9±0.1$</td>
<td>8.7±0.1$</td>
</tr>
<tr>
<td>BERK + 5% arginine</td>
<td>3.7±0.6§</td>
<td>21.3±1.7</td>
<td>33.4±6.4</td>
<td>22.4±0.7</td>
<td>8.9±0.5</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 5 in each group (C57BL and BERK-Hemi mice) and 10 in each group (BERK mice). Hct, hematocrit; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; Hemi, hemizygous. *P < 0.001 vs. C57BL mice; †P < 0.04 vs. untreated BERK-Hemi mice; ‡P < 0.001 vs. untreated BERK mice; §P < 0.001 vs. untreated BERK-Hemi mice.
Plasma PGE2 Determination

Plasma was collected from heparinized blood, and PGE2 was determined using PGE metabolite enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI).

Statistical Analysis

Comparisons were made using Student’s t-test. Where tests for normality failed, or Bartlett’s test for homogeneity of variance showed significant difference in the standard deviations, nonparametric tests, such as Kruskal-Wallis test for ANOVA or the Wilcoxon two-sample test, were used. P < 0.05 was considered significant.

RESULTS

In the present studies, we evaluated the effect of arginine on vasodilator enzymes and microvascular parameters in the cremaster muscle microcirculatory bed of sickle mice. In addition, we measured cell-free plasma hemoglobin and oxidative stress (i.e., nitrotyrosine).

Arginine Has No Effect on eNOS Expression, But Reduces Expression of Non-NO Vasodilators

eNOS. Among untreated groups, eNOS expression (eNOS-to-actin ratio) in BERK and BERK-Hemi mice showed no significant differences compared with control C57BL mice (n = 3 each). Arginine supplementation caused 52 and 31% decreases in eNOS expression in BERK-Hemi and BERK mice, respectively (P < 0.001 and <0.25) (Fig. 1A).

Non-NO Vasodilators

COX enzymes. Western blots (Fig. 1, B and C) and densitometric analysis (Fig. 2A) revealed that, among untreated groups (n = 3 each), COX-2 expression showed approximately fourfold increase in BERK mice (P < 0.01) and 2.8-fold increase in BERK-Hemi mice (P < 0.05) compared with C57BL mice. Arginine had no effect on COX-2 expression in C57BL mice, but caused 52 and 43% decreases in COX-2 expression in BERK-Hemi and BERK mice, respectively (P < 0.01 and P < 0.05 vs. respective untreated controls, n = 3 each; Figs. 1, B and C, and Fig. 2A). Notably, the expression of constitutively expressed COX-1 showed no differences among untreated or arginine-treated groups (Fig. 1D).

Plasma PGE2 levels. As shown in Fig. 2B, the overall pattern of PGE2 levels followed the level of COX-2 expression depicted in Fig. 2A. COX-1 expression in BERK mice was not different from that in C57BL or BERK-Hemi mice, implying that the marked increase in PGE2 levels in BERK mice was due to COX-2 induction. Consistent with its effect on COX-2 expression (Fig. 2A), arginine caused 56% decrease in PGE2 levels in BERK mice (means ± SE: from 1,225 ± 72 to 536 ± 130 pg/ml, P < 0.004), but had no effect on PGE2 levels in C57BL and BERK-Hemi mice.

HO-1. In three BERK mice examined, HO-1 expression showed variable increases (2.3-, 4.2-, and 11.6-fold) compared with the averaged control values (Fig. 1E). Arginine had no appreciable effect on HO-1 expression in C57BL mouse. Importantly, arginine treatment of BERK mice resulted in marked 42–45% reduction in HO-1 expression. In BERK-Hemi mice (Fig. 1E), HO-1 expression was elevated by 7.6-fold, which was reduced by 40% after arginine treatment.

Arginine Improves Microvascular Parameters in Sickle (BERK) Mice

Arteriolar diameters. Consistent with our laboratory’s previous studies (19), BERK mice (n = 4) showed significant 28.4–42.5% dilation of cremasteric arterioles (branching orders: A2, A3, and A4) compared with controls (P < 0.01, n = 4; Fig. 3A). Arginine caused 20–42% diameter increases in the arterioles in C57BL (P < 0.01) and BERK-Hemi mice (P < 0.05–0.01, n = 4). In contrast, in BERK mice (n = 6), arginine resulted in a significant decrease in the diameter of A2 arterioles (P < 0.003), but had no effect on A3 and A4 diameters. It should be noted that the postarginine arteriolar diameters in each group were remarkably similar for any given arteriolar order, as depicted by horizontal lines in Fig. 3A.

Hemodynamic parameters. The effect of arginine on microvascular flow parameters was evaluated in A2 arterioles. In particular, the reduction in the A2 diameter in arginine-treated BERK mice caused 17.5% increase (P < 0.01) in the wall...
Hemi and BERK mice showed greater PGE2 levels compared with C57BL decreases, respectively, reduced expression of COX-2 in BERK-Hemi and BERK mice (52 and 43% BERK mice showing the maximal increase. Arginine caused a markedly BERK-Hemi and BERK mice (*P < 0.05 and **P < 0.01, respectively), with BERK mice showing the maximal increase. Arginine caused a markedly reduced expression of COX-2 in BERK-Hemi and BERK mice (52 and 43% decreases, respectively. +P < 0.01 and ++P < 0.038). B: untreated BERK-Hemi and BERK mice showed greater PGE2 levels compared with C57BL mouse (*P < 0.05 and **P < 0.0001, respectively). Arginine caused marked 56% decrease in BERK mice (+P < 0.0004 vs. untreated BERK).

Shear rate (s⁻¹) (Fig. 3B). As previously reported (3), untreated BERK mice also showed maximal Q (nl/s) in A2 arterioles compared with control groups (Fig. 3C). Likewise, A2 diameter increase in arginine-treated C57BL and BERK-Hemi mice was associated with a greater Q (P < 0.01 and <0.05 vs. respective untreated controls); the resulting Q was not different from that in untreated BERK mice. In contrast, in arginine-treated BERK mice, A2 diameter decrease resulted in 27% decline in Q compared with the untreated group (P < 0.027), indicating a trend toward normalization (Fig. 3C).

Arginine Improves Vascular Reactivity to NO-Mediated Vasoactive Stimuli

Response to ACh, an endothelium-dependent vasodilator. Topical ACh (10⁻⁶ M) caused 76.6 and 73.3% increases, respectively, in arteriolar (A3) diameters (means ± SE) of untreated C57BL control (24.1 ± 2.2 to 42.4 ± 4.0 μm, P < 0.001, n = 5) and BERK-Hemi mice (22.6 ± 3.0 to 37.6 ± 3.2 μm, P < 0.001, n = 5) (Fig. 4A). In contrast, untreated BERK mice showed an attenuated response to ACh, as revealed by only a 7.3% increase in the arteriolar diameter (29.2 ± 2.3 to 32.1 ± 2.8 μm, P < 0.01, n = 5), in agreement with our laboratory’s previous findings (19). Arginine had no significant effect on arteriolar responses to ACh in C57BL and BERK-Hemi mice. In contrast, ACh caused 43.5% increase in the arteriolar diameter of arginine-treated BERK mice (32.7 ± 2.6 to 47.0 ± 4.0 μm, P < 0.0001; Fig. 4A).

Response to SNP, a NO donor. As shown in Fig. 4B, topical SNP (10⁻⁶ M) caused 81.1 and ~65% increases in arteriolar (A3) diameters, respectively, in untreated C57BL controls (23.6 ± 2.1 to 42.3 ± 3.6 μm, P < 0.001, n = 5) and BERK-Hemi mice (20.0 ± 2.5 to 32.7 ± 3.5 μm, P < 0.00001, n = 5). In contrast, untreated BERK mice showed distinctly blunted response, as evidenced by only 15% increase in the diameter (29.4 ± 2.6 to 33.3 ± 2.2 μm, P < 0.00001 vs. C57BL and BERK-Hemi mice), which is consistent with our laboratory’s reported findings (19). In marked contrast, SNP caused ~60% increase in arteriolar diameters in arginine-treated BERK mice (30.5 ± 2.3 to 48.2 ± 4.4 μm, P < 0.0002, n = 6; Fig. 4B).

Response to L-NAME. To investigate the effect of arginine on NOS activity, we measured the response of the MAP to intravenous infusion of L-NAME. As depicted in Fig. 4C, L-NAME caused significant increases (133–155%) in MAP in untreated C57BL control (n = 5) and BERK-Hemi mice (n = 3) (P < 0.01 and 0.05, respectively, vs. pre-L-NAME values),
but resulted in an attenuated (i.e., 14% increase) response in untreated BERK mice \((n = 5)\). The baseline MAP in BERK mice was also markedly lower than those for C57BL and BERK-Hemi mice (each \(P < 0.001\)). Arginine had no effect in C57BL and BERK-Hemi mice. In marked contrast, l-NAME caused 43% increase in MAP in arginine-treated BERK mice \((P < 0.001)\).

**Arginine Reduces Hemolysis**

As shown in Table 1, cell-free plasma hemoglobin was significantly elevated in BERK mice compared with controls (C57BL and BERK-Hemi) mice (each \(P < 0.001\)), which is comparable with our laboratory’s previous studies \((19)\). In BERK mice, arginine resulted in marked >50% decrease in plasma hemoglobin \((P < 0.001)\), but had no significant effect in controls (Table 1). However, the reduced hemolysis in arginine-treated BERK mice was not associated with significant changes in Hct, %reticulocytes, and MCH. This is because a decrease in micromolar cell-free hemoglobin is not expected to affect millimolar whole blood hemoglobin levels, as the amount of hemoglobin in plasma is smaller by more than a factor of 1,000 than the hemoglobin present in red cells in whole blood. On the other hand, arginine caused a significant increase in Hct \((P < 0.04)\) and a decrease in reticulocyte counts \((P < 0.03)\) in BERK-Hemi mice. Untreated BERK mice showed lower Hct, higher reticulocyte counts, and lower MCH compared with untreated C57BL and BERK-Hemi mice, as previously reported \((3)\).

**Arginine Increases Plasma Citrulline and NOx Levels**

We monitored plasma citrulline and NOx levels as measures of NO production. Plasma arginine levels \((\mu\text{mol/l}, \text{means \pm SE})\) in untreated BERK mice \((36 \pm 8 \mu\text{mol/l}, \text{n} = 9)\) were \(-58%\) lower than the C57BL baseline values \((85 \pm 5 \mu\text{mol/l}, \text{P} < 0.0001, \text{n} = 14)\). Also, as depicted in Table 2, the lowest citrulline levels were monitored in untreated BERK mice \((P < 0.03\) vs. untreated C57BL mice). Following arginine treatment, citrulline levels rose 67 and 51% in BERK-Hemi and BERK mice, respectively, and the increase was significant for BERK mice \((P < 0.005)\). Notably, citrulline levels in arginine-treated BERK mice were not significantly different from the baseline values of C57BL mice. Because arginine can also be metabolized into ornithine by the enzyme arginase, we determined plasma ornithine levels (Table 2). Untreated BERK mice showed elevated plasma ornithine values compared with C57BL mice \((P < 0.03)\), suggesting increased arginase activity in these mice. Arginine caused increases in ornithine levels in C57BL \((P < 0.0001)\) and BERK-Hemi mice \((P < 0.005)\), but the increase in BERK mice was not significant (Table 2).

NOx levels showed 65 and 80% decreases, respectively, in BERK-Hemi and BERK mice compared with C57BL mice (each \(P < 0.0001\)), indicating minimal NO bioavailability in BERK mice (Table 2). Arginine caused 48 and 34.5% increases in NOx levels, respectively, in C57BL and BERK-Hemi mice compared with untreated groups \((P < 0.001\) and \(P < 0.01)\). In contrast, arginine resulted in greater than twofold decreases in these mice. Arginine-treated BERK mice showed markedly improved responses to acetylcholine (ACh) and sodium nitroprusside (SNP) \((P < 0.0001\) vs. C57BL and BERK-Hemi mice, \(+P < 0.0001\) vs. untreated BERK mice). In contrast to significant increases in untreated C57BL and BERK-Hemi mice \((P < 0.005)\), NOx levels showed 65 and 80% decreases, respectively, in BERK-Hemi and BERK mice compared with untreated groups \((P < 0.001\) and \(P < 0.01)\). In contrast, arginine resulted in greater than twofold decreases in these mice. Arginine-treated BERK mice showed markedly improved responses to acetylcholine (ACh) and sodium nitroprusside (SNP) \((P < 0.0001\) vs. C57BL and BERK-Hemi mice, \(+P < 0.0001\) vs. untreated BERK mice).

**Table 2. Effect of arginine on plasma citrulline, ornithine, and NOx levels in C57BL, BERK-Hemi, and BERK mice**

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Citrulline, (\mu\text{mol/l})</th>
<th>Ornithine, (\mu\text{mol/l})</th>
<th>NOx, (\mu\text{mol/l})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
<td>5% Arginine</td>
<td>Untreated</td>
</tr>
<tr>
<td>C57BL</td>
<td>56±4 (15)</td>
<td>76±12 (11)</td>
<td>55±4 (15)</td>
</tr>
<tr>
<td>BERK-Hemi</td>
<td>52±3 (4)</td>
<td>87±13 (6)</td>
<td>67±4 (5)</td>
</tr>
<tr>
<td>BERK</td>
<td>43±3 (9)†</td>
<td>65±8 (6)*</td>
<td>85±14 (9)†</td>
</tr>
</tbody>
</table>

*Values are means \(\pm SE\); nos. in parentheses are no. of mice. NOx, nitric oxide metabolites. \(*P < 0.01\) vs. respective untreated mice; \(*P < 0.0001\) vs. untreated C57BL; †\(P < 0.03\) vs. C57BL.
increase in NOx levels in BERK mice compared with the untreated group ($P < 0.002$). This is in agreement with our laboratory’s earlier studies, wherein we compared NOx levels in NY1DD transgenic sickle (mild pathology) and BERK mice (but not BERK-Hemi mice) after arginine supplementation (5).

### Arginine Reduces Nitrotyrosine Expression

eNOS dysfunction has been associated with enhanced peroxynitrite (ONOO$^-$) formation in several pathological states, including BERK mice (16, 45), which reduces NO bioavailability. Hence, we examined the effect of arginine on nitrotyrosine formation, which is the result of protein nitration by ONOO$^-$. Western blots revealed two major bands of nitrated proteins, corresponding approximately to 60 and 26 kDa (Fig. 5A). Densitometric analysis (Fig. 5, B and C) revealed that, among untreated groups, tyrosine nitration of $\sim$60- and 26-kDa proteins was markedly increased in BERK-Hemi and BERK mice (60-kDa protein: 2.9- and 4.6-fold increase, respectively, $P < 0.01$; 24-kDa protein: 4- and 5.6-fold, $P < 0.05$ and 0.01, respectively; $n = 4$ each) compared with C57BL controls ($n = 4$). Arginine had no effect on protein nitration in C57BL mice. In marked contrast, arginine caused distinct 53–71% decreases in tyrosine nitration of the 60- and 26-kDa proteins in BERK and BERK-hemi mice ($P < 0.05–0.01$) (Fig. 5, B and C), indicating a decreased oxidative stress.

### NO Bioactivity: the Effect of Hemolysis and Oxidative Stress

Next, we analyzed our data whether hemolysis and/or oxidative stress affected the observed arteriolar diameter response (Fig. 4B) to SNP, a NO donor, as a measure of NO bioactivity. As shown in Fig. 4A, linear regression of the data showed a strong relationship between plasma hemoglobin levels and the mean arteriolar diameter increase (%) in response to SNP. A similar strong relationship was noted when arteriolar diameter response to SNP was plotted against levels of nitrotyrosine formation (Fig. 4B). Thus untreated BERK mice with highest plasma hemoglobin (7.7 ± 0.5 μmol, see Table 1) or nitrotyrosine formation showed minimal arteriolar diameter increase in response to SNP, whereas, after arginine treatment, SNP caused a marked 60% increase in the diameter, which was associated with reduced plasma hemoglobin values (3.7 ± 0.6 μmol) and nitrotyrosine formation.

### DISCUSSION

We show that arginine supplementation of transgenic-knockout sickle (BERK) mice significantly ameliorates several manifest abnormalities. First, in this mouse model of SCD, arginine reduces the expression of non-NO vasodilator enzymes, such as COX-2 that produces PGE2 and HO-1 that produces CO. Second, the decreased expression of non-NO vasodilators is associated with reduced vasodilation and improved vascular reactivity to NO-mediated vasoactive stimuli. Finally, arginine reduces hemolysis and oxidative stress, both of which are implicated in consumption and/or inactivation of NO, as well as in altered microvascular responses.

Notably, in BERK mice, arginine significantly reduces the expression of non-NO vasodilator enzymes COX-2 and HO-1, which are likely to contribute to the altered vascular tone and reactivity in untreated BERK mice. COX-2 is induced under the conditions of chronic hypoxia and oxidative stress.

ONOO$^-$ has been implicated in the induction of COX-2 (24). COX-2 induction in BERK mice is associated with severalfold increase in PGE2 production, which will contribute to vasodilation in these mice. Higher PGE2 values probably contribute to the impaired vasoreactivity in BERK mice and in untreated BERK-Hemi and BERK mice. Additionally, arginine reduces COX-2 expression and PGE2 levels, probably by decreasing oxidative stress. The second non-NO vasodilator enzyme HO-1, induced in response to hemolysis, hypoxia, and oxidative stress, catalyzes degradation of heme to biliverdin/

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**Fig. 5.** Effect of arginine on nitrotyrosine formation. A: BERK mice showed maximal tyrosine nitration of both $\sim$60- and 26-kDa proteins. Arginine caused marked decreases in tyrosine nitration of 60- and 26-kDa proteins in BERK and BERK-hemi mice ($P < 0.05–0.01$) (Fig. 5, B and C), indicating a decreased oxidative stress.
bibilirubin and carbon monoxide (34, 43). Hence, HO-1 is also a marker of hemolysis with vasodilatory properties via generation of carbon monoxide. In fact, the markedly reduced HO-1 expression in arginine-supplemented BERK mice is associated with decreased hemolysis. We propose that the suppression of COX-2 and HO-1 expression following arginine treatment is a consequence of increased NO bioavailability that results in improved vascular tone and reactivity.

The protective effect of arginine on microvascular regulation in BERK mice is evident by the response of resistance vessels (arterioles). Untreated BERK mice show significant arteriolar dilation, increased Q (see Fig. 3), and systemic hypotension (see Fig. 4C), which is associated with induction of non-NO vasodilator enzymes, COX-1 and HO-1. While arginine causes significant increases in arteriolar diameters in C57BL and BERK-Hemi mice, it results in a significant reduction in arteriolar diameters in BERK mice, accompanied by marked decrease in the flow and reduced expression of non-NO vasodilators, suggesting a trend toward normalization. Notably, the resulting arteriolar diameters after arginine supplementation are strikingly similar in controls and BERK mice, suggesting that increased NO bioavailability may contribute to vascular homeostasis in BERK mice (see Fig. 3A).

Furthermore, arginine treatment in sickle mice causes improved arteriolar diameter response to NO-mediated vasoactive stimuli. In contrast to robust vasodilatory effects of ACh and SNP in untreated control C57BL and BERK-Hemi mice, untreated BERK mice show blunted responses to these stimuli, probably due to inactivation and/or consumption of NO by oxidants and/or plasma hemoglobin (19, 36). On the other hand, reduced oxidative stress and hemolysis in arginine-treated BERK mice are accompanied by markedly improved arteriolar diameter response to ACh and SNP. The improved response to ACh indicates a greater availability of substrate arginine. Also, distinctly blunted blood pressure response in untreated BERK mouse to L-NAME indicates impaired NO/NOS-mediated control of vascular tone. In contrast, in arginine-treated BERK mice, L-NAME causes marked 43% increase in blood pressure. These results show distinctly improved vascular reactivity to NO-mediated vasoactive stimuli in arginine-supplemented BERK mice.

An important finding of the present studies with implications for NO bioavailability is the pronounced >50% decrease in cell-free plasma hemoglobin in arginine-treated BERK mice (see Table 1), which is expected to reduce NO inactivation by cell-free ferrous (oxy) hemoglobin (36). Arginine may reduce hemolysis by preventing oxidative stress-induced hemolysis of red cells. Oxygen radicals have been implicated in hemolysis of red cells in several pathological states, including β-thalassemia and SCD (13, 21). Arginine therapy of sickle patients (0.1g/kg, 3 times/day for 1 mo) has been reported to increase red cell GSH, a key red cell antioxidant that may decrease red cell oxidative stress and hemolysis (27). Similarly, arginine causes significant increase in tissue GSH in BERK mice (5). The second and potentially concurring mechanism may involve inhibition of red cell Gardos channel by arginine, as reported by Romero et al. in transgenic sickle mouse (39), which will reduce dense cell formation and improve red cell deformability. Arginine supplementation of sickle transgenic mice reduces plasma endothelin-1 (ET-1) levels (38). ET-1 has been shown to stimulate the Gardos channel (37), and reduction of ET-1 may partially account for the reduction in GSH and ET-1 activity in arginine-supplemented sickle mice. In humans, administration of clotrimazole, a Gardos channel inhibitor, did not reduce plasma lactate dehydrogenase (a surrogate marker of hemolysis) significantly (4). Further work will be needed to unravel the roles of ET-1 and its receptors during arginine supplementation.

Arginine significantly decreases oxidative stress, as evidenced by reduced nitrotyrosine expression in BERK mice. Excessive generation of free oxygen radicals can result in increased OONO$^-$ formation and nitration of tyrosine residues of proteins. Although other mechanisms of nitrotyrosine formation are now recognized (41, 45), in this model, formation of superoxide and OONO$^-$ appears to be the most straightforward explanation. Also, decreased oxidative stress following arginine treatment may inhibit the reported uncoupling of eNOS (16, 45) and thereby enhance NO synthesis.

The reduced hemolysis and oxidative stress in arginine-treated BERK mice result in enhanced NO production, as indicated by greater than twofold increase in NOx levels and >50% increase in citrulline levels, the latter approaching control (C57BL) levels. On the other hand, in BERK mice, arginine does not result in significant increase in plasma ornithine, a measure of arginase activity. Although mouse red cells lack arginase, the observed increase in ornithine levels in arginine-treated control mice suggests that arginase derived from other mouse tissues, such as endothelium (6, 44), may be involved in this conversion.
Although future studies will be required to delineate the relationship between hemolytic rate and oxidative stress in terms of cause and effect, the present studies show that reducing hemolysis and oxidative stress distinctly result in improved NO bioavailability. We find that the arteriolar diameter response to SNP, a NO donor, is strongly correlated with hemolytic rate (and nitrotyrosine formation), i.e., the greater the hemolytic rate, the lesser the arteriolar diameter increase in response to SNP (see Fig. 6, A and B). Also, elevated levels of cGMP caused by HO-1 induction (32) in response to excess plasma heme may contribute to the blunted vasodilator effect of SNP in BERK mice. The reduced hemolysis in arginine-treated BERK mice is expected to reduce NO inactivation and result in a greater response to SNP.

Although pharmacokinetics of arginine supplementation may vary among mammalian species (42), the increased NO bioavailability after arginine treatment, as noted in our studies, is more compatible with results obtained in sickle patients, wherein arginine was orally administered at a dose of 0.1 g/kg, three times a day (27, 29).

In conclusion, we show for the first time that arginine exerts a range of protective effects in sickle mice, which include improved microvascular function, reduced expression of non-NO vasodilators, decreased hemolysis and oxidative stress, and increased NO bioavailability. These results demonstrate that increasing NO bioavailability by therapeutic agents such as arginine is a promising strategy in the management of SCD.

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GRANTS

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