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Arginase Inhibition Reverses Endothelial Dysfunction, Pulmonary Hypertension and Vascular Stiffness in Transgenic Sick Cell Mice

Jochen Steppan, MD*,

Department of Anesthesiology and Critical Care Medicine, Johns Hopkins Medical Institution, Baltimore, Maryland

Huong T Tran, PhD*,

Department of Anesthesiology and Critical Care Medicine, Johns Hopkins Medical Institution, Baltimore, Maryland

Valeriani R Bead, MD,

Department of Medicine, Division of Cardiology, Johns Hopkins Medical Institution, Baltimore, Maryland

Corresponding Author: Lakshmi Santhanam, PhD, Department of Anesthesiology and Critical Care Medicine, The Johns Hopkins University, School of Medicine, 720 Rutland Ave, Ross 1150, Baltimore, MD 21205, Phone: 443-287-6718, Fax: 410-955-0994, lsantha1@jhmi.edu.

*Both authors contributed equally to the work.

The authors declare no conflicts of interest.

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Name: Jochen Steppan, MD

Contribution: This author helped design the study, analyze the data, and write the manuscript.

Attestation: Jochen Steppan has seen the original study data, reviewed the analysis of the data, approved the final manuscript, and is the author responsible for archiving the study files.

Name: Huong T Tran, PhD

Contribution: This author helped design the study, conduct the study, and analyze the data.

Attestation: Huong T Tran has seen the original study data, reviewed the analysis of the data, and approved the final manuscript.

Name: Valeriani R Bead, MD

Contribution: This author helped design the study, conduct the study, and analyze the data.

Attestation: Valeriani R Bead has seen the original study data, reviewed the analysis of the data, and approved the final manuscript.

Name: Young Jun Oh, MD

Contribution: This author helped conduct the study.

Attestation: Young Jun Oh has seen the original study data and approved the final manuscript.

Name: Gautam Sikka, MD

Contribution: This author helped conduct the study.

Attestation: Gautam Sikka has seen the original study data and approved the final manuscript.

Name: Trinity J Bivalacqua, MD, PhD

Contribution: This author helped design the study.

Attestation: Trinity J Bivalacqua approved the final manuscript.

Name: Arthur Burnett, MD

Contribution: This author helped design the study.

Attestation: Arthur Burnett has seen the original study data and approved the final manuscript.

Name: Dan E Berkowitz, MD

Contribution: This author helped design the study, analyze the data, and write the manuscript.

Attestation: Dan E Berkowitz has seen the original study data, reviewed the analysis of the data, and approved the final manuscript.

Name: Lakshmi Santhanam, PhD

Contribution: This author helped design the study, analyze the data, and write the manuscript.

Attestation: Lakshmi Santhanam has seen the original study data, reviewed the analysis of the data, and approved the final manuscript.

This manuscript was handled by: Avery Tung, MD

Young Jun Oh, MD,

Department of Anesthesiology and Critical Care Medicine, Johns Hopkins Medical Institution, Baltimore, Maryland; Department of Anesthesiology and Pain Medicine, Yonsei University, College of Medicine, Seoul, South Korea

Gautam Sikka, MD,

Department of Anesthesiology and Critical Care Medicine, Johns Hopkins Medical Institution, Baltimore, Maryland

Trinity J Bivalacqua, MD, PhD,

Department of Urology, Johns Hopkins Medical Institution, Baltimore, Maryland

Arthur L Burnett, MD,

Department of Urology, Johns Hopkins Medical Institution, Baltimore, Maryland

Dan E Berkowitz, MD, and

Department of Anesthesiology and Critical Care Medicine, Johns Hopkins Medical Institution, Baltimore, Maryland

Lakshmi Santhanam, PhD

Department of Anesthesiology and Critical Care Medicine, Johns Hopkins Medical Institution, Baltimore, Maryland

Abstract

Background—In sickle cell disease (SCD), hemolysis results in the release and activation of arginase, an enzyme that reciprocally regulates nitric oxide (NO) synthase activity and thus, NO production. However, simply supplementing the common substrate L-arginine fails to improve NO bioavailability. In this study, we tested the hypothesis that arginase inhibition would improve NO bioavailability and thereby attenuate systemic and pulmonary vascular endothelial dysfunction in transgenic mice with SCD.

Methods and Results—We studied 5 month-old transgenic sickle cell (SC) mice and age-matched wild-type (WT) controls. SC mice were treated with the arginase inhibitor, 2(S)-Amino-6-BoronoHexanoic acid (ABH; ~400 µg/d) for four weeks or left untreated. Vascular arginase activity was significantly higher at baseline in untreated SC mice compared to WT controls (SC vs. WT, 346 ±69.3 vs. 69 ±17.3 pmol urea/mg protein/minute; p=0.0043; n = 4–5 animals per group). ABH treatment may significantly decrease arginase activity to levels near WT controls (SC + ABH 125.2 ±17.3 pmol urea/mg protein/minute, p=0.0213). Aortic strips from untreated SC mice showed decreased nitric oxide (NO) and increased reactive oxygen species (ROS) production (NO: fluorescence rate 0.76 ±0.14 vs. 1.34 ±0.17 RFU/s, p = 0.0005 and ROS: fluorescence rate 3.96 ±1.70 vs. 1.63 ±1.20 RFU/s, p = 0.0039; n = 3–5 animals per group). SC animals treated with ABH for 4 weeks demonstrated NO (fluorescence rate: 1.16 ±0.16) and ROS (fluorescence rate: 2.02 ±0.45) levels comparable to age-matched WT controls (n = 3–5 animals per group). The maximal endothelial-dependent vasorelaxation response to acetylcholine was impaired in aortic rings from SC mice compared to WT (57.7 ±8.4% vs. 80.3 ±11.0%; p=0.02; n = 6 animals per group). The endothelial-independent response was not different between groups. In SC mice, the right ventricular cardiac output index, and end-systolic elastance were similar (4.60 ±0.51 vs 2.9 ±0.85 ml/min/100g and 0.89 ±0.48 vs 0.58 ±0.11 mmHg/µl), while the pulmonary

vascular resistance index and right ventricular end systolic pressure were higher (2.9 ± 0.28 vs 5.5 ± 2.0 mmHg $\times$ min/ μ l/100g and 18.9 ± 1.1 vs 23.1 ± 4.0 mmHg; $n = 8$ animals per group). Pulse wave velocity (a measure of arterial stiffness) was higher in SC mice compared to WT (3.74 ± 0.54 vs. 3.25 ± 0.21 m/s; $n = 20$ animals per group), arginase inhibition for four weeks significantly reduced the vascular SC phenotype to one similar to WT animals ($p=0.0009$).

Conclusion—Arginase inhibition improves NO bioavailability and thereby attenuates systemic and pulmonary vascular endothelial dysfunction in transgenic mice with SCD. Therefore, arginase is a potential therapeutic target in the treatment of cardiovascular dysfunction in SCD.

INTRODUCTION

The hereditary blood disorder, sickle cell disease (SCD) results from a single nucleotide mutation in the β -globin gene, which leads to the loss of red blood cell deformability. Diseased red blood cells are susceptible to changes of their shape (sickling), which can be precipitated by hypoxia, stress, and hypothermia. The relatively stiff cells can no longer undergo the required shape change necessary to transit the microvasculature, resulting in vessel occlusion, ischemia, and pain crises. Over the past several decades, our understanding of the complex pathophysiology of vaso-occlusion, which is the hallmark of SCD, has improved dramatically. Originally, it was believed that stiff (sickled) red blood cells resulting from polymerization of hemoglobin S were the primary contributor to the clinical pathophysiology of SCD. Research in human subjects and in animal models, however, has identified dysregulated vascular pathways, known as sickle vasculopathy, which features severe hemolytic anemia leading to scavenging of nitric oxide (NO).⁽¹⁾ This diminished NO bioavailability in turn leads to pulmonary hypertension, cutaneous leg ulceration, priapism, ischemic stroke as well as renal insufficiency, systolic hypertension and overall endothelial dysfunction.^(2–4) NO has been described as a mediator of SCD and it has been demonstrated previously that the disruption of endothelial regulation of vasoreactivity is a likely contributor to sickle vasculopathy and pulmonary hypertension.⁽⁵⁾ Moreover, NO was shown to inhibit the adherence of sickle cells to the vascular endothelium in SCD.⁽⁶⁾ Thus, in principle, restoring NO bioavailability should therefore result in a marked improvement in vascular function in SCD. It is therefore critical to either verify or refute the role of NO bioavailability and its pharmacological manipulation in SCD in preclinical models, which will in future lay the foundation to develop specific therapeutic agents that impact pathways affecting NO resistance in SCD.

Arginase, an enzyme that is released from red blood cells, competes with NO synthase (NOS) for their common substrate, L-arginine. Increased arginase activity consequently results in decreased L-arginine availability for endothelial NOS (eNOS).⁽⁷⁾ Increased arginase activity in patients with SCD has been suggested by prior studies. Morris et al. showed that a lower L-arginine to ornithine ratio (which corresponds to higher arginase activity) was associated with more severe pulmonary hypertension and a higher mortality in human subjects.⁽⁸⁾ In order to determine whether L-arginine supplementation can recover eNOS activity, Little et al. conducted a phase I/II clinical trial in which exogenous L-arginine was administered to patients with SCD. However, L-arginine supplementation resulted in increased plasma arginine and ornithine levels without changing citrulline levels,

suggesting that most of the arginine administered is metabolized by arginase rather than NOS.⁽⁹⁾ Furthermore chronic L-arginine exposure has been demonstrated to increase endothelial cell arginase, essentially “adding fuel to the fire.”⁽¹⁰⁾

There is increasing evidence that arginase reciprocally regulates eNOS function in the vasculature. We and others have shown that arginase activation contributes to an age-related decrease in endothelial NO and subsequent vascular endothelial dysfunction and stiffness. Moreover, in the absence of sufficient substrate availability (L-arginine), eNOS produces superoxide instead of NO.⁽¹¹⁾ Arginase thereby contributes to eNOS uncoupling in cellular and animal models of atherosclerosis by limiting substrate availability for eNOS.^(7,12,13) Importantly, we have shown that arginase inhibition improves vascular endothelial NO bioavailability, decreases reactive oxygen species (ROS) production and enhances endothelial-dependent vasodilation.⁽¹⁴⁾ Importantly there are distinct intracellular pools of L-arginine, of which only some can be accessed by NOS.^(15,16) Thus, failure in previous trials to improve vascular mechanics by L-arginine supplementation might be either due to the inaccessibility of L-arginine pools to NOS or due to L-arginine consumption by arginase. One approach to improve L-arginine bioavailability to NOS would be to limit L-arginine consumption by arginase. We therefore examined whether arginase inhibition can improve global NO bioavailability, and thereby attenuate vascular endothelial dysfunction, pulmonary pressures and vascular stiffness in transgenic mice with SCD.

METHODS

Animals

Transgenic sickle cell (SC) mice were obtained from Lawrence Berkeley National Labs.⁽¹⁷⁾ These mice express exclusively human sickle hemoglobin and all mouse hemoglobin genes are deleted. Age-matched C57Bl/6 mice were used as wild type (WT) controls. Forty SC animals (4–5 month-old males) were randomized into 2 groups with 20 animals per group: untreated SC mice (SC), SC mice treated with 2(S)-Amino-6-BoronoHexanoic acid (SC +ABH). WT animals were used as controls. Noninvasive pulse wave velocity (PWV) measurements were acquired in all of these mice. At the end of the experimental period, the 20 animals from each cohort were randomized for end-point measures as follows: six animals were used for biochemistry, six for vasoreactivity (organ bath), and eight for cardiac catheterization. Each animal in the treatment group consumed approximately 400 µg of ABH (Calbiochem, Germany) daily in their drinking water for four weeks. All mice were maintained on standard rodent diet and all studies were approved by the Animal Care and Use Committees of the Johns Hopkins Medical Institute.

Arginase activity

Mouse aorta was frozen in liquid nitrogen and pulverized. Resulting tissue powder was combined 1:4 (wt:vol) with ice-cold lysis buffer (1× RIPA containing protease inhibitors (PMSF, leupeptin, aprotinin)) and homogenized. The homogenate was centrifuged at 12,000g for 10 minutes, and the supernatant recovered for an arginase activity assay as described previously.⁽¹⁸⁾

Production of reactive oxygen species and nitric oxide

ROS were measured *ex vivo* as described previously, using DHE fluorescence (Pierce Protein Biology Products, Thermo Scientific, Rockford, IL).⁽¹⁹⁾ NO production was measured *ex vivo* using DAF FM-DA (Molecular Probes, Eugene, 3B. OR), a NO-sensitive fluorescent dye; as described previously.⁽²⁰⁾

Vasoreactivity studies

Aortic rings were prepared and suspended in organ chambers as previously described.⁽¹⁴⁾ Vessels were pre-constricted with phenylephrine (10^{-6} mol/L) (Sigma, St. Louis, MO) or PGF 2α (10^{-8} mol/L) (Sigma, St. Louis, MO). Endothelium-dependent and independent responses were determined with acetylcholine (ACh, 10^{-9} to 10^{-5} mol/L) and sodium nitroprusside (10^{-9} to 10^{-5} mol/L) (Sigma, St. Louis, MO), respectively.

Cardiac catheterization

The procedure was conducted as described previously.⁽²¹⁾ In brief, all animals were anesthetized with 0.08 – 0.15 mL of 300 mg/mL urethane, 0.05 mL of 2 mg/mL etomidate, and 0.02 μ L of 1.5 g/mL morphine and placed on a heating pad. Rectal temperature was measured throughout the experiment and maintained at baseline. The duration of the experiments was 45–60 min, which is well below the duration of the anesthetics used (90 minutes) and therefore, we did not adjust the depth of anesthesia during the experiments. A tracheotomy tube was placed and connected to a ventilator (Hugo Sachs Elektronik Type 845 Mouse Minivent) set at a respiratory rate between 110 and 120 breath per minutes at 300 μ L of air per breath. As described previously, the apex of the heart was accessed through the diaphragm using a lateral incision below the xyphoid process.^(22,23) Using a 27½ gauge needle, the tip of a 1.4 F SPR-839 Millar micro-conductance catheter was introduced into the right ventricle via a retrograde approach and then in to the left ventricle via a similar approach. Data were recorded using a Millar Aria 1 PV Conductance System and Chart 5 from AD Instrument PowerLab. To obtain pressure volume loops and chamber-specific hemodynamic variables, manual occlusion of the isolated inferior vena cava was used to initiate a transient reduction in preload.

Pulse wave velocity

PWV was measured noninvasively using high frequency Doppler (Indus Instruments, Tx) as previously described.⁽²⁴⁾ PWV is measured at two points along the aorta with one point in the upper thoracic cavity and one point in the lower abdominal cavity. PWV is calculated as the separation distance divided by the pulse transit time between the two points.

Statistical analysis

Data are reported as mean $\pm$ SD (standard deviation). Arginase activity, NO activity, ROS production, cardiac catheterization, and PWV were analyzed using one way ANOVA followed by Tukey's test to obtain p values for one way ANOVA and multiplicity adjusted p values for each comparison. For the vasoreactivity experiments a two-way-ANOVA followed by Bonferroni test for multiple comparisons was used, with a total of 27 effective groups. A p value of less than 0.01 was used as the criterion for statistical significance. The p-values of

the ANOVA are presented in results. The *post hoc* p values for all direct comparisons are presented in the Figure legends.

RESULTS

Arginase activity

Arginase activity was significantly higher in the aortic homogenates of untreated SC mice compared to WT controls. ABH treatment resulted in a marked reduction in arginase activity in the SC mice compared to the untreated SC cohort (SC vs. WT vs. SC+ABH: 341 ± 69.3 vs. 90.1 ± 17.3 vs. 120 ± 17.3 pmol urea/mg protein/minute, $p=0.0007$ by one way ANOVA; $n = 4-5$ animals per group; Figure 1).

Nitric oxide and reactive oxygen species

Aortic strips from untreated SC mice showed significantly decreased NO production compared to WT controls. Arginase inhibition with ABH resulted in increased NO production in SC mice compared to the untreated SC cohort (rate of NO production SC: 0.76 ± 0.14 ; WT: 1.34 ± 0.17 ; SC+ABH: 1.16 ± 0.16 RFU/s; $n = 3$ animals per group; Figure 2A) ($p = 0.0006$ by one-way ANOVA). Conversely, the rate of ROS production was higher in the aortic strips from untreated SC mice compared to age-matched WT controls. Arginase inhibition decreased ROS production in SC mice compared to the untreated SC group (rate of ROS production: SC: 3.96 ± 1.70 ; WT: 1.63 ± 1.20 ; SC+ABH: 2.02 ± 0.45 RFU/s; $n = 5$ per group; Figure 2B) ($p=0.02$ by one way ANOVA).

Vasoreactivity studies and endothelial function

Vasodilatation in response to increasing concentrations of ACh was similar in all groups. Only the maximal endothelial-dependent vasodilatory response to ACh (10^{-5} M) decreased in SC mice when compared with WT controls ($p=0.02$ by two-way ANOVA with Bonferroni's multiple comparisons test). ABH treatment restored maximal endothelial-dependent vasodilation in SC mice to that of WT mice (Figure 3A). The endothelial-independent response to the NO donor sodium nitroprusside was similar in all three groups (EC₅₀ in SC: 1.1×10^{-8} , WT: 8.5×10^{-9} , and SC+ABH: 1.3×10^{-8} ; maximal relaxation in SC: $98 \pm 5.8\%$, SC+ABH: $95 \pm 2.6\%$, and WT: $95 \pm 5.9\%$; $n = 6$; Figure 3B).

Right heart catheterization

Pulmonary vascular resistance index (PVRI) and right ventricular end systolic pressure (RVESP) may be significantly elevated in SC mice compared to WT animals (PVRI: 5.5 ± 1.9 vs. 2.9 ± 0.28 mmHg $\times$ min/ μ l/100g $p=0.004$; and RVESP: 23.1 ± 4.0 vs. 18.9 ± 1.13 mmHg; $p=0.03$; Figure 4). Right ventricular cardiac output index (RVCOI) and right ventricular end-systolic elastance (RVEes) in SC mice were similar to WT animals (4.60 ± 0.51 vs. 2.9 ± 0.85 ml/min/100g; $p=0.10$; and 0.58 ± 0.11 vs. 0.89 ± 0.48 mmHg/ μ l; $p=0.018$; Figure 4 and 5). Treatment with ABH for four weeks restored PVRI and RVESP, to levels similar to WT animals (PVRI: 3.4 ± 1.4 mHg $\times$ min/ μ l/100g, RVESP: 20.1 ± 3.1 mmHg; Figure 4 and 5). ABH treatment may elevate RVCOI in the SC animals when compared with untreated SC animals with no effect on RVEes (RVCOI: 5.1 ± 2.5 ml/min/100g, $p = 0.03$ vs. SC; RVEes: 0.79 ± 0.31 mmHg/ μ l; Figure 4 and 5).

Pulse wave velocity and arterial stiffness

PWV, a measure of vascular stiffness, was higher in untreated SC mice than in WT controls. SC mice treated with ABH for four weeks had significantly reduced PWVs compared to untreated SC mice and were no different from WT controls (PWV in m/s: SC: 3.74 ± 0.54 ; WT: 3.25 ± 0.21 m/s; SC+ABH: 3.06 ± 0.31 ; $p=0.0009$ by one way ANOVA; $n = 20$; Figure 6).

DISCUSSION

Loss of global NO and overall endothelial dysfunction is now well established in SCD. In this study, we show that arginase activity is significantly increased in SC mouse aorta compared to WT while NO production is reduced. More importantly, we believe this is the first demonstration that arginase inhibition improves vascular function and reverses the vascular phenotype of SCD. These findings are in accordance with prior studies demonstrating not only arginase upregulation, but more specifically upregulation of arginase II as well as ornithine decarboxylase antizyme mRNA in platelets of SC patients.^(25,26) Moreover we demonstrated an attenuated endothelial-dependent vasorelaxation response to ACh in SC mice.

Physiological changes in SC vasculopathy include increased pulmonary resistance, increased right ventricular systolic pressures and higher PWV suggesting stiffer vessels. These changes are recapitulated well in the SC mouse. One way to improve NO signaling downstream of NO in human subjects is through phosphodiesterase (PDE) inhibition. Interestingly, modest increases in plasma cyclic guanosine monophosphate and citrulline levels and improved pulmonary pressures as well as 6-minute walk test distance were observed in patients receiving sildenafil.⁽⁹⁾ A subsequent double-blind study in patients with pulmonary hypertension and decreased exercise tolerance that compared placebo to sildenafil, was terminated after enrollment of 74 patients because there were more hospitalizations for pain in the sildenafil group, no difference in the 6-minute walk distance, and a trend to increased pulmonary hypertension in the sildenafil group.⁽²⁷⁾ This is likely because the PDE5 inhibitor does not address the issue of eNOS uncoupling, and the high oxidative stress and low NO bioavailability in the vasculature remain unchanged. Indeed, if PDE5 inhibitors are to work, at least some bioavailable NO needs to be present to activate soluble guanylate cyclase. However because these studies were performed in human subjects, only plasma levels of arginase were evaluated and endothelial arginase was not examined.⁽⁸⁾

In this study, treatment of SC mice with the arginase inhibitor ABH for 4 weeks decreased arginase activity and restored pulmonary pressures, resistance, and PWV to a level comparable with age-matched WT animals. Vasodilation in response to increasing concentrations of ACh in aortic rings was similar in all cohorts. This could, in part, have been due to the age of the mice used in this study, with older mice demonstrating impaired vasoreactivity. Additionally, the increased PWV in these mice suggests that the *in vivo* environment with its fully functional mechanical and biochemical signaling mechanisms along with sickling cells may be required to observe alterations in vasoreactivity; a feature that is lost in the *in vitro* study. Finally, endothelial dysfunction may be dependent on the

vascular bed. Indeed, to our knowledge the only observation to date of endothelial dysfunction in this SC mouse model has been in the pulmonary circulation *in vivo*⁽²⁸⁾.

Overall, these findings support prior studies that have proposed that arginase may be an important target in SCD-related vasculopathy and provide evidence for the use of arginase inhibitors for the treatment of sickle cell vasculopathy.^(28,29) Although there are currently no specific arginase inhibitors available for human use, in 2007 it was demonstrated that chloroquine, a drug used as an antimalarial or antirheumatoid medication, inhibits arginase in a dose-dependent manner including in sickle erythrocytes.⁽³⁰⁾ Continued efforts to identify and characterize specific and selective arginase inhibitors are needed to further elucidate the therapeutic index of arginase in human subjects.

It is widely accepted that hemolytic rates and release of free hemoglobin into the circulation closely correlate with scavenging of NO. Two mechanisms can account for this loss of NO: first, cell-free hemoglobin can rapidly react with NO and second, the released arginase 1 can consume L-arginine to further compromise substrate availability for NOS.⁽²⁹⁾ However given that arginase 2 is also upregulated in the endothelium of SC mice, there appears to be an endothelial contribution to SC vasculopathy that is independent from red blood cell hemolysis. Future studies will focus on separating the contribution of each arginase isoform (arginase 1 in the red blood cells vs. arginase 2 in the endothelium) to the SC phenotype.

Limitation

This study was performed in a rodent model with some experiments conducted *ex vivo*. The physiological experiments were performed using mice under general anesthesia with the chest cavity exposed to ambient pressure and air, and the lungs ventilated with positive pressure. This could have resulted in a change of pressures, flows and volumes measurements, and might not have been identical to awake and intact animals. Furthermore placement of the catheter is difficult in small rodents and was done by visual recognition of pressure waveforms by a single provider without verification by other imaging modalities. Because methodological variations may limit the generalizability of our results, we used a more stringent *post hoc* p-value < 0.01 as statistically significant. In the present study, it was not possible to delineate whether the detrimental effects of arginase in SCD are due to intracellular arginase 2 of the affected cells or due to the amount of arginase 1 liberated from the erythrocytes by hemolysis. These mechanisms can be distinguished by measuring endothelial and plasma NO and arginase in SC mice and their WT controls using pharmacological inhibition of each isoform.

Conclusion

Two important findings of this study are: 1) increased arginase activity contributes to endothelial dysfunction, pulmonary hypertension, and vascular stiffness in transgenic SC mice; and 2) chronic treatment with an arginase inhibitor, ABH, attenuates systemic and pulmonary vascular dysfunction in transgenic SC mice. Thus arginase is a potential novel therapeutic target in SC vasculopathy.

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Figure 1. Arginase activity in mouse aortic homogenates

Arginase activity is higher in sickle cell mice (SC; n = 5) compared to wild type controls (WT; n = 4; p=0.0009). Arginase inhibition (2(S)-Amino-6-BoronoHexanoic acid [ABH]; n = 5) in SC mice decreases arginase activity, to a level comparable to WTs (p=0.69 compared to WT and p=0.002 compared to SC; (**post hoc* p-value < 0.01)).

Figure 2. Nitric oxide levels and reactive oxygen species

A) Nitric oxide production is decreased in sickle cell (SC) aorta compared to wild types (WT; $p=0.009$). Arginase inhibition with 2(S)-Amino-6-BoronoHexanoic acid (ABH) increases nitric oxide production ($n = 3$ animals per group; $p=0.39$ compared to WT and $p=0.05$ compared to SC). B) Rates of reactive oxygen species (ROS) production are higher in SC mice compared to WT ($p=0.03$). ABH treatment decreases this in SC mice ($n = 5$ animals per group; $p=0.87$ compared to WT and $p=0.07$ compared to SC); (**post hoc* p -value < 0.01).

Figure 3. Vasoreactivity and endothelial function

A) Sick cell mice (SC) have a reduced maximal endothelial dependent vasodilatory response to acetylcholine (ACh) compared to wild types (WT, $p=0.18$), which becomes apparent at an ACh dose of 10^{-5} M. Inhibition of arginase restores endothelial-dependent vasodilation ($p>0.99$ compared to WT). B) There is no difference in the endothelial-independent response (sodium nitroprusside) between the groups ($n = 6$ animals per group, $p>0.99$ in all comparisons).

Figure 4. Right heart catheterization

A) Pulmonary vascular resistance index (PVRI) and B) right ventricular end systolic pressure (RVESP) were elevated in sickle cell (SC) mice compared to wild types (WT). C) Right ventricular cardiac output index (RVCOI) and right ventricular end-systolic elastance (RVEes) were similar in SC mice compared to WT. Treatment with ACh for 4 weeks restored PVRI, RVESP, RVCOI, and RVEes (n = 8 animals per group, *post hoc* p-values: PVRI: WT vs SC p=0.004, WT vs SC+2(S)-Amino-6-BoronoHexanoic acid (ABH) p=0.76, SC vs SC+ABH p=0.19; RVESP: WT vs SC p=0.03, WT vs SC+ABH p=0.70, SC vs SC+ABH p=0.13; RVCOI: WT vs SC p=0.10, WT vs SC+ABH p=0.80, SC vs SC+ABH p=0.03; RVEes: WT vs SC p=0.18, WT vs SC+ABH p=0.83, SC vs SC+ABH p=0.44; *p<0.01).

Figure 5. Right ventricular pressure volume loops

Representative traces of right ventricular pressure volume loops in A) wild type, B) sickle cell, and C) sickle cell mice after treatment with 2(S)-Amino-6-BoronoHexanoic acid (ABH).

Figure 6. Pulse wave velocity

Pulse wave velocity is higher in sickle cell (SC) mice than in wild types (WT, $p=0.09$). SC mice treated with 2(S)-Amino-6-BoronoHexanoic acid (ABH), have significantly reduced PWV to a level that is not statistically different from WT ($n = 20$ animals per group; $p=0.73$ compared to WT and $p=0.001$ compared to SC; $*p < 0.01$).