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Increasing nitric oxide bioavailability fails to improve collateral vessel formation in humanized sickle cell mice

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Abstract

Sickle cell disease (SCD) is associated with repeated bouts of vascular insufficiency leading to organ dysfunction. Deficits in revascularization following vascular injury are evident in SCD patients and animal models. We aimed to elucidate whether enhancing nitric oxide bioavailability in SCD mice improves outcomes in a model of vascular insufficiency. Townes AA (wild type) and SS (sickle cell) mice were treated with either L-Arginine (5% in drinking water), L-NAME (N(ω)-nitro-L-arginine methyl ester; 1 g/L in drinking water) or NO-generating hydrogel (PA-YK-NO), then subjected to hindlimb ischemia via femoral artery ligation and excision. Perfusion recovery was monitored over 28 days via LASER Doppler perfusion imaging. Consistent with previous findings, perfusion was impaired in SS mice (63±4% of non-ischemic limb perfusion in AA vs 33±3% in SS; day 28; $P<0.001$; n=5–7) and associated with increased necrosis. L-Arginine treatment had no significant effect on perfusion recovery or necrosis (n=5–7). PA-YK-

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Author Contributions

C.V.L and W.R.T performed study concept and design and writing and revision of paper; H.W.J, L.A.B, and D.R.A performed development of methodology; C.V.L, L.A.B, H.S, L.H, G.J, and J.H provided acquisition, analysis and interpretation of data; D.R.A, L.A.B and H.W.J provided technical support and all authors contributed to review and revision of the paper.

Ethics Approval and Consent to Participate

These studies did not involve human participants, data or tissue and therefore ethical approval was not required.

Conflict of Interest

All authors declare that they have no conflicts of interests in relation to this work.

Competing Interests: The authors declare no competing financial interests.

NO treatment led to worsened perfusion recovery (19±3 vs 32±3 in vehicle-treated mice; day 7; P<0.05; n=4–5), increased necrosis score (P<0.05, n=4–5) and a 46% increase in hindlimb peroxynitrite (P=0.055, n=4–5). Interestingly, L-NAME worsened outcomes in SS mice with decreased in vivo lectin staining following ischemia (7±2% area in untreated vs 4±2% in treated mice, P<0.05, n=5). Our findings demonstrate that L-arginine and direct NO delivery both fail to improve postischemic neovascularization in SCD. Addition of NO to the inflammatory, oxidative environment in SCD may result in further oxidative stress and limit recovery.

Introduction

Sickle cell disease (SCD) is the most common hemoglobinopathy worldwide and is associated with significant morbidity and mortality. Although stem-cell transplantation and gene therapy approaches are promising, there remains a need for affordable and readily available treatments to improve and extend patient lives, particularly given disease prevalence in low socio-economic regions. Most of the clinical consequences of SCD such as stroke, pain crises, renal failure and retinopathy, arise from repeated episodes of vascular occlusion and vessel injury[1–4]. We recently reported that humanized SCD mice have impaired recovery from vascular injury[5]. Using hindlimb ischemia (HLI) as a model of vascular insufficiency, we demonstrated that SCD mice lacked functional collateral vessel formation and exhibited excessive and unresolved inflammation. Recovery was improved with either neutrophil depletion or antioxidant therapy, demonstrating an important contribution of non-resolving neutrophilic inflammation and oxidative stress[5]. However, the potential contribution of decreased nitric oxide bioavailability in these processes was not investigated and may be a central underlying mechanism for poor responses to vessel ischemia in SCD.

A role for nitric oxide signaling in collateralization is evident in several studies of eNOS deficient mice[6,7]. It is well-established that SCD is associated with dysregulated nitric oxide (NO) signaling via multiple mechanisms. Chronic intravascular hemolysis leads to release of free hemoglobin that scavenges NO. This hemoglobin, along with released free heme and iron, creates a highly oxidative, proinflammatory environment. Under chronic oxidative stress, endothelial nitric oxide synthase (eNOS) inhibitors such as asymmetric dimethylarginine (ADMA) increase in expression[8] while the important co-factor tetrahydrobiopterin (BH4) is degraded[9]. This leads to eNOS uncoupling and production of superoxide rather than NO, further exacerbating oxidative stress. Together, these processes create a vicious cycle of oxidative stress and reduced NO bioavailability in SCD. Additionally, arginase released from lysed RBCs consumes the eNOS substrate arginine, with increased plasma arginase demonstrated in patients and mouse models of SCD[4,10–13]. Consequently, SCD patients have impaired NO-dependent blood flow and chronic inflammation[14–16].

Over recent years, therapies aimed at restoring NO bioavailability have been explored in multiple clinical trials for SCD. Inhaled NO therapy is the most extensively tested but, at least for acute chest syndrome and recovery from VOC, it has proved largely ineffective, likely due to the short-acting nature of the treatment[17,18]. However, L-arginine

supplementation as an approach to increase NO bioavailability has shown more promising results[19]. In transgenic SCD mice L-arginine was shown to significantly increase NO levels and decrease oxidative stress and lipid peroxidation[20]. Early encouraging clinical results for low dose L-arginine demonstrated increased NO metabolites (NOx), decreased oxidative stress, improved liver function and decreased hemolysis in SCD patients[21–23]. However, a larger scale phase II clinical trial of low dose oral L-Arginine failed to demonstrate an increase in NOx or any clinical benefits[24]. A more recent study suggested this dosing was sub-therapeutic for SCD patients and tested a higher intravenous dose, administered to patients after admission for VOC. This randomized placebo-controlled trial of 56 children demonstrated a >50% reduction in opioid used and pain scores following treatment[25]. These findings are further supported by a recent SCD trial for high dose oral arginine conducted in Nigeria[26]. In a smaller cohort, intravenous arginine treatment also improved mitochondrial function and limited oxidative stress[27]. These promising data suggest a potential beneficial effect of restoring arginine and NO levels on SCD outcomes. A NIH-funded, multi-center clinical trial for L-Arginine treatment in SCD (Sickle Cell Disease Treatment with Arginine Therapy (STarT), www.clinicaltrials.gov identifier NCT04839354) is currently underway and will provide more definitive information on the potential of this therapy for SCD.

In normal physiology, NO has many vasoprotective properties including facilitating flow-mediated vasodilation and eliciting anti-inflammatory and anti-thrombotic effects[28,29]. In the setting of ischemia, eNOS-NO pathways have been shown to play important roles in collateral vessel formation and perfusion recovery. $eNOS^{-/-}$ mice subjected to hindlimb ischemia have severely impaired recovery from HLI with reduced arteriogenesis and increased ischemic injury and necrosis[7]. This is attributed to impaired vascular endothelial growth factor (VEGF) signaling in response to ischemia, reduced flow-mediated dilation of collateral vessels, and regulation of cell cycle genes leading to reduced proliferation of collateral vessel cells[6,7]. The mechanistic role of eNOS and NO in revascularization in SCD response to ischemia has not been investigated. In this study we aimed to determine whether enhancing NO generation improves recovery of SCD mice in a model of vascular insufficiency. We proposed that restoration of NO bioavailability, either by L-arginine supplementation or local injection of an NO-producing hydrogel (PA-YK-NO), would improve collateral vessel formation and perfusion recovery.

Materials and Methods

Animals

The humanized Townes SS (sickle cell) mice were an original generous gift of Dr Townes (University of Alabama, Birmingham). The Townes mice, hα/hα::βA/βS, hα/hα::−383 γ-βA/-1400 γ-βS (Jax No.013071) are on a mixed C57BL/6:129 background, and colonies were established, maintained, and bred in-house at the Emory University Department of Animal Resources. Female wildtype mice (genotype AA) and littermate SS mice (genotype SS) between 8 and 12 weeks old were used in this study. All female mice were used in this study as they have impaired hindlimb ischemia outcomes as compared to males[30]. Mice were housed and cared for in agreement with the guidelines approved by the Emory University Institutional Animal Care and Use Committee. Serum was obtained via

cardiopuncture following euthanization for analysis of NO metabolites, amino acid content and arginase activity.

Hindlimb Ischemia and necrosis grading

Mice were anesthetized with 2% isoflurane through a nose cone. Using aseptic technique, a unilateral incision was made over the left thigh and the superficial femoral artery and vein were ligated with 6–0 silk proximal to the caudally branching deep femoral artery. A second ligation was made just proximal to the branching of the tibial arteries. The artery and vein were excised between the ligation points with the femoral nerve intact. The skin was closed with monofilament suture and mice allowed to recover on a heated pad. Mice received buprenorphine (0.1 mg/kg, subcutaneous) just prior to surgery.

Necrosis was graded on a scale from 0 to 5 based on the level of visible necrosis and amputation. 0= no necrosis, 1= one necrotic toe, 2= two or more necrotic toes, 3= foot necrosis, 4= leg necrosis and/or foot autoamputation, 5= autoamputation of leg. SCD mice were monitored daily and a score of 4 or above resulted in immediate euthanization.

LASER Doppler Perfusion Imaging

LASER (light amplification by stimulated emission of radiation) Doppler perfusion imaging (LDPI) was performed 1, 7, 14, 21 and 28 days after HLI surgery as previously described[31]. Mice were anesthetized with 2% isoflurane and scanned with the LDPI system (Moor Instruments). Equivalent regions of interest were defined on the generated perfusion heatmap for the mouse hindlimbs and mean perfusion (arbitrary perfusion units) quantified for the whole, proximal and distal limb regions. Perfusion in the ischemic leg was normalized to the non-ischemic leg (perfusion ratio).

L-Arginine and L-NAME treatment

L-Arginine (50 g/L or 100 g/L; Sigma-Aldrich A8094) or the NOS inhibitor L-N^G-nitro arginine methyl ester (L-NAME; 1 g/L; Sigma-Aldrich N5751) was added to the drinking water and replaced daily from the day of surgery for 7–28 days as indicated.

NO hydrogel delivery to hindlimb

Low (0.9 μM NO) or high (1.8 μM NO) [NO]-generating hydrogel PA-YK-NO was generated as previously described[32–34]. In brief, peptide amphiphile (PA)-YK was synthesized and self-assembled by mixing PA-YIGSR (endothelial cell adhesive ligand coupled with an MMP-2 degradable sequence) and PA-KKKKK (NO donor poly-lysine) in a 9:1 ratio and then reacted with NO gas to synthesize PA-YK-NO. PA-YK-NO was then mixed 1:1 with calcium chloride for gelation just prior to injection[33]. For injection in our model, 75 μl PA-YK-NO was combined with 75 μl $CaCl_2$ within a 1 ml syringe and quickly injected into the hindlimb after surgery at 6 sites surrounding the excision (6 injections of ~25 μl, 150 μl total per mouse), just prior to suturing. Vehicle treated mice received PBS with $CaCl_2$ in the same volumes. PA-YK-NO releases NO continuously. NO concentrations are shown to peak and plateau after 2–3 days followed by consistent NO release for at least 28 days[34].

In vivo lectin staining

Hindlimb vessels were imaged using in vivo lectin staining as previously described[5]. Briefly, 100 ul of 1 mg/ml fluorescein isothiocyanate-conjugated griffonia simplicifolia lectin I (FITC-lectin; Cat No. FL-1101; VectorLabs) was injected intravenously and allowed to circulate for 10 minutes before mice were euthanized and hindlimb muscles dissected. Tissues were fixed in 10% buffered formalin overnight, incubated in 30% sucrose for 24 hours, and embedded in optimal cutting temperature compound for snap-freezing. Frozen tissue was cryosectioned (50 μm), mounted with Vectashield with DAPI (4',6-diamidino-2-phenylindole, dihydrochloride; Cat No. H-1200; VectorLabs) and imaged on a confocal laser scanning microscope. FITC-lectin staining of perfused vessels was quantified using ImageJ software as percent fluorescence per tissue area. An average of five fields of view were taken for each animal and analysis was performed blinded.

Serum NO metabolite (NOx) assay

Serum was collected from AA or SS mice at day 7, 14 or 28. Total NOx concentration in serum samples was determined using the Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical, Cat No. 780001) according to the manufacturer's instructions. In brief, samples were filtered through 10 kDA molecular weight cut-off filters (Amicon Ultra Centrifugal Filters, Millipore) by centrifugation and 40 μl taken per replicate, diluted with 40 ul of assay buffer. Nitrate reductase treatment was used to convert all nitrate in the samples to nitrite. Total nitrite was detected using Griess reagents with absorbance measured at 540 nm on a plate reader. Sample values were plotted against a nitrite standard curve to determine concentration.

Arginase assay

Arginase activity in serum and hindlimb was measured using the Arginase Activity Assay Kit (Sigma-Aldrich, Cat no. MAK112) according to the manufacturer's instructions. This kit is non-selective and thus represents activity of both arginase I and arginase II. Endogenous serum urea was depleted using 10 kDA molecular weight cut-off filters prior to assay. For the hindlimb, tissue was homogenized and diluted in assay buffer for detection within the linear range of the assay. Colorimetric detection of urea to indicate arginase activity was performed on a plate reader with absorbance measured at 430 nm. Activity (U/L) was calculated using blanks and standards following the equation provided by the manufacturer.

Western blot

Western blots were performed to detect the expression of argininosuccinate synthase 1 (ASS1), ornithine transcarbamylase (OTC), argininosuccinate lyase (ASL) and ornithine decarboxylase (ODC) in liver and kidney. Total protein extracts (20 μg) were separated on 10% SDS-PAGE and transferred (1 hr, 4°C) to nitrocellulose membranes (100 Volts). After blocking (5% non-fat milk in 0.1% Tween Tris buffered saline), the nitrocellulose membranes were incubated with the following primary antibodies directed against the following proteins: ASS1 (1:500), ASL (1:200), ODC (1:200), and OTC (1:100; Santa-Cruz, CA), β-actin (1:3000) and PKG (1:1000, Cell Signaling). Membranes were then incubated

with HRP-conjugated secondary antibodies (BioRad, 1 hr, room temperature) and the signal was detected by enhanced chemiluminescence (Pierce, Rockford, IL).

Polymerase Chain Reaction

Total RNA was extracted from homogenized thoracic aortas using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNase-free DNase (Qiagen) was used to remove any contaminating DNA. RNA was reverse transcribed into cDNA and purified with QiaQuick (Qiagen). Gene expression was quantified on an Applied Biosystems StepOnePlus Real-Time PCR System. IL-6, TNFα and 18S Quantitect Primer Assays were purchased from Qiagen. Gene expression was normalized to housekeeping gene 18S and expressed relative to the average untreated wildtype (AA) value using the comparative cycle threshold (Ct) method with the formula: Fold change= $2^{-\Delta\Delta Ct}$.

Serum amino acid detection

For amino acid analysis of the serum (100 μl), the Phenomenex EZ:faast™ amino acid analysis kit was used according to the manufacturer's protocol After the solid phase extraction step via a tip packed with a sorbent that binds amino acids, sample amino acids were derivatized to modify the carboxyl and amino groups, and then extracted using a liquid/liquid extraction method. The derivatized amino acids in the organic phase were evaporated to dryness, resuspended, and then analyzed by high-performance liquid chromatographic-tandem mass spectrometric/mass spectrometric methods in the electrospray positive ionization mode. A binary gradient elution of mobile phases A (0.2% formic acid containing 5 mM ammonium acetate) and B (methanol, containing 0.2% formic acid and 5 mM ammonium acetate) were programmed at 0.4 mL/min.

Hydrogen peroxide detection

Extracellular H_2O_2 from ischemic hindlimb muscles were measured using the Amplex Red assay kit (Invitrogen; Cat No. A22188), according to manufacturer's instructions as previously described[5]. Fluorescence was detected on a fluorescence plate reader (Ex/Em= 530/580 nm) with background fluorescence subtracted. Fluorescence was normalized to dry tissue weight.

Peroxynitrite detection

Extracellular ONOO- production was measured in ischemic hindlimb muscles using the Amplite™ Fluorimetric Peroxynitrite Quantification Kit (AAT Bioquest; Cat No. 16316) according to the manufacturer's instructions. Briefly, muscles were dissected and collected in PBS on ice just prior to assay. DAX-J2™ Green 99 500X stock was prepared in DMSO and diluted in assay buffer for 1X working solution. 300 μl of working solution was added to wells of a 24 well plate and tissue quickly transferred into the solution. The mixture was incubated at room temperature for 10 minutes, protected from light. Solution was transferred to a 96 well plate in triplicate and fluorescence detected on a fluorescence plate reader (Ex/Em= 490/530 nm) with background fluorescence subtracted. Fluorescence was normalized to dry tissue weight.

Statistical Analysis

Necrosis grading data is presented as median and interquartile range and analyzed by Mann-Whitney (2 groups) or Kruskal-Wallis (3 groups) tests. All other results are expressed as mean ± SEM, tested for normality and parametric tests selected for analysis. Prism (Graphpad Software, La Jolla, CA) was used for statistical analysis. Power analysis was performed to determine n for significance $P<0.05$ with a power of >0.9 for primary endpoints (LDPI, necrosis). Time course perfusion recovery experiments were analyzed by 2-way repeated measures ANOVA, followed by Sidak's post hoc test. All other experiments were analyzed by student's unpaired t-test (2 groups) or 1-way ANOVA (3 or more groups) with Sidak's post hoc test. Corrections were made for multiple comparisons.

Results

L-Arginine supplementation fails to improve recovery from hindlimb ischemia in SCD

In agreement with previous reports[12,13], SS mice had a more than 4-fold increase in serum arginase activity as compared to wildtype AA controls (4.5 ± 2.6 in AA vs 21.2 ± 1.4 in SS, $P<0.05$, n=4; Figure 1A). There was also a trend for increased arginase in both non-ischemic (NIL) and ischemic limbs (IL) of these mice at 7 days post-surgery (Figure 1B). To confirm that our L-Arginine supplementation protocol (5% in drinking water) increased arginine bioavailability, we assessed serum arginine and global arginine bioavailability ratio (GABR). Despite significant variability in the treated groups, the treatment did appear to increase both measures in AA mice (Arginine: 374.2 ± 20.8 to 917 ± 165 μM, P=0.09; GABR: 0.77 ± 0.03 to 2.83 ± 0.7, P= 0.08, n=5–7; Figure 1C–D) and arginine in SS mice (361.8 ± 88 to 836.9 ± 219 μM, P=0.13, n=5–7; Figure 1C). Although mean GABR increased from 0.96 ± 0.3 to 2.26 ± 0.6 in SS mice, these results were highly variable and did not approach statistical significance (Figure 1D). Contrary to several reports of decreased serum arginine in SCD[21,35], SS mice did not have decreased arginine or GABR at baseline (Figure 1C–D). Despite increased arginine levels following treatment, increased serum NO metabolites (NOx) were observed in AA (5.32 ± 0.2 to 15.86 ± 5.2 μM, $P<0.01$, n=4–6) but not in SS mice (5.94 ± 0.5 to 5.39 ± 0.4 μM; n=6–7; Figure 1E), suggesting that the arginine delivered to SS mice did not increase synthesis of NO.

Given that L-arginine supplementation has been reported to have antioxidant effects in SCD[20,22], we investigated its effects on hindlimb reactive oxygen species (ROS) at D7 (peak inflammation) following HLI. As we previously reported, SS mice had significantly (3-fold) elevated hydrogen peroxide compared to AA mice. L-arginine supplementation attenuated this increase (Figure 1F). We also demonstrated 2-fold increased peroxynitrite in SS mice, however levels of this ROS remained elevated in the L-arginine group (Figure 1G). Consistent with our previous findings, necrosis scores were increased (0.1±0.1 in AA vs 2.4±0.7 in SS mice; day 28; $P<0.05$, n=5–7) and perfusion was significantly impaired in SS mice (63±4% of non-ischemic limb perfusion in AA vs 33±3% in SS mice; day 28; $P<0.001$; n=5–7). Importantly, at the completion of the 28-day study, L-arginine-treated SS mice had necrosis scores and perfusion recovery equivalent to untreated SS mice and substantially worse than AA controls (Figure 1H–J). As 5% L-Arginine supplementation did not increase NOx levels, we also tested a higher concentration of

L-arginine (10% in drinking water). However, this dose failed to improve perfusion but resulted in gastrointestinal symptoms and weight loss (Supplementary Figure 1). Taken together, these results suggest that L-arginine supplementation is not a reliable method of increasing NO bioavailability in SCD mice and, despite a modest effect to limit hydrogen peroxide generation, it does not improve recovery from hindlimb ischemia.

Unexpected circulating ornithine levels are not explained by altered L-arginine cycling enzymes

In our assessment of GABR ([arginine]/[citrulline] + [ornithine]) we noticed unexpected results for ornithine. We anticipated increased ornithine in SS mice due to augmented arginase activity, but contrary to our prediction, we observed a decrease (267.8 ± 46 μM in AA vs 145.5 ± 20 μM in SS, $P<0.05$, n=7; Figure 2B). In addition, SS mice had increased citrulline (198.7 ± 25 μM in AA vs 282.3 ± 46 μM in SS, $P<0.05$, n=7; Figure 2A) and their arginine levels were higher than expected, given the dramatic increase in arginase activity (Figure 1). We proposed that these observations could be explained by dysregulated expression of enzymes involved in arginine cycling (Figure 2I). Specifically, increased conversion of ornithine to citrulline by ornithine transcarbamylase (OTC), decreased conversion of citrulline to arginine by argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL). Additionally, ornithine decarboxylase (ODC) could be converting the increased ornithine to putrescine. It is well known that the liver and kidney are important sites of arginine regulation and the kidney in particular is an important site for conversion of citrulline to arginine. In addition, both organs are dysfunctional in SCD. We found no differences in liver expression of ASS1 or ASL (Supplementary Figure 2). In the kidney, we observed no significant changes in protein expression of ASS1, ASL or ODC in SS mice (Figure 2C–G) but did observe a modest increase in OTC expression in SS mice (0.34 ± 0.04 in AA vs 0.53 ± 0.08 in SS, $P=0.06$, n=6; Figure 2H) that could contribute to increased conversion of ornithine to citrulline.

NO hydrogel injections worsen recovery from hindlimb ischemia in SCD

To directly test the potential for NO to improve response to ischemia, we next tested a method of sustained NO delivery to the hindlimb. NO-generating hydrogel (PA-YK-NO) or vehicle ($CaCl_2$) was injected into SS hindlimbs immediately following surgery, prior to suturing the leg. We confirmed increased NO metabolites in the hydrogel group in serum (Figure 3A). Unfortunately, the assay was not sensitive enough to reliably detect NO metabolites in hindlimb homogenates. Nonetheless this systemic effect suggests an increase would have been evident in the hindlimb where the hydrogel was delivered. Contrary to our hypothesis that NO delivery would improve collateral vessel formation, the hydrogel led to decreased perfusion (32±3% in vehicle vs 19%±3 in PA-YK-NO-treated mice at D7 for whole limb, $P<0.05$, n=4–5; Figure 3B) and increased limb necrosis (1.8±0.4 in vehicle-treated vs 3±0.8 in PA-YK-NO-treated mice; $P<0.05$, n=4–5; Figure 3D) at day 14. We also tested a high [NO]-PA-YK-NO hydrogel but this resulted in further worsening of necrosis and required several mice to be euthanized due to limb autoamputation (Supplementary Figure 3A–B). Additionally, the low [NO]-PA-YK-NO hydrogel was tested using a different time course (2 days prior to surgery and again at day 12 post surgery) to assess whether pre-treatment and established NO generation in the hindlimb was required to improve perfusion.

This strategy had no effect on perfusion recovery in AA or SS mice (Supplementary Figure 3C). As the ischemic limb is a site of inflammation, we proposed that the NO-hydrogel was worsening outcomes via increased peroxynitrite generation. Indeed, we found a 46% increase in peroxynitrite levels in the hindlimbs of PA-YK-NO-treated SS mice at D14 (P=0.055, n=4–5; Figure 3E). Hydrogen peroxide levels were unchanged by the treatment (Figure 3F).

L-NAME treatment worsens recovery from hindlimb ischemia in SCD

Following the observation that increasing NO levels appeared to worsen rather than improve outcomes, we wanted to investigate the role of eNOS in SCD mice. As eNOS is reportedly uncoupled in SCD, its inhibition would be expected to limit superoxide generation and dampen inflammation, as was demonstrated in cerebral venules of aged SCD mice[36]. Treatment with the NOS inhibitor L-NAME (1g/L in drinking water), resulted in decreased serum NO metabolites (Figure 4A), and increased severity of necrosis (Figure 4B). Both of these observations indicate the presence of functional eNOS in SS hindlimb that has a protective effect on recovery. To further characterize the effect of L-NAME on collateralization, we injected FITC-labelled lectin antibody to visualize vessels in the hindlimb 7 days after surgery. SS mice treated with L-NAME had decreased lectin staining, 4.6 ± 0.7 % area compared to 7.2 ± 0.7 % in control SS mice, suggesting further impairment in collateral vessel formation following treatment ($P<0.05$, n=5; Figure 4C). Interestingly, this observation of a detrimental effect of NOS inhibition in the peripheral vasculature is contrary to observations in the aorta where 7-day L-NAME treatment of non-surgery mice decreased TNFα expression (4.5 ± 0.3-fold to 2.7 ±0.8-fold of AA expression, $P<0.05$) and prevented an increase in IL-6 (2.1 ± 0.3-fold vs 1.4 ± 0.06-fold of AA expression; n=4–6 Figure 4D–E) suggesting a pro-inflammatory effect of uncoupled eNOS in the SS aorta but not in the hindlimb.

Discussion

Though it is a commonly assumed contributor to SCD, low NO bioavailability is a controversial hypothesis in SCD pathology with varying reports on NO levels and efficacy of NO-related therapies. L-arginine therapy, both oral supplementation and acute IV treatment, have been explored in patients with VOC with some promising results in small clinical trials[19]. However, contrary to the generally accepted theory that these pathways are protective in SCD, in our study of humanized SCD mice, we demonstrate that L-arginine and NO fail to confer protection in a model of vascular insufficiency. L-arginine supplementation was not sufficient to increase circulating NOx levels and its modest antioxidant effect did not lead to any improvement in response to vessel injury. We also demonstrate that NO delivery to ischemic tissue does not improve, and in fact worsens, outcomes. These observations suggest a limitation of therapies that target NO bioavailability in SCD and highlight that although restoring NO levels may be beneficial in some contexts, it should be approached cautiously in cases of acute vessel injury and ischemia where the addition of NO to the inflammatory, oxidative environment may result in further oxidative stress and limit recovery.

L-Arginine supplementation

We found that in the Townes humanized SCD mouse, there was a strong trend for L-arginine treatment to increase circulating arginine, but not NOx levels, despite this treatment successfully enhancing NOx levels in littermate controls. Current data on L-arginine and NOx levels in SCD patients at steady state and following L-arginine treatment is highly variable. Generally, studies in adults show decreased steady state circulating arginine[11,21,23]. In children however, arginine and NO deficiencies are only observed during vaso-occlusive episodes[37]. Furthermore, steady state NOx has been shown to be decreased[21,38] and increased[23,39] in SCD compared to controls in different studies. In response to L-arginine treatment, most trials of SCD patients with VOC report increased NOx[21,23]. However, a paradoxical decrease in circulating NOx in steady state SCD patients who receive L-arginine demonstrates altered arginine metabolism in SCD depending on disease severity[23] and low dose oral arginine, likely the most relevant dosing strategy to our study, required long term (4 month) treatment before increased NOx could be observed[40].

As with human studies, there is considerable heterogeneity in outcomes for arginine and NOx levels in SCD mice. Elevated nitrite and cGMP levels were recently reported in both Townes and BERK mice[39] in contrast to others that report a decrease[13,20,35]. At odds with our findings for baseline circulating arginine, decreased plasma arginine has been shown in both BERK and Townes SCD mice[13,35]. Similarly, decreased baseline NOx that is increased following L-arginine supplementation is reported in BERK and NY1DD mice[20] but was not observed in our mice. These discrepancies could be due to differences in mouse background or arginine delivery and detection methods. Notably, the controls used in our studies express human hemoglobin genes and are on a mixed C57BL/6:129 background, whereas controls used for BERK mice are wildtype C57BL/6 and have higher NOx levels. Our findings that L-arginine fails to enhance NOx levels in SCD are not entirely unexpected and support the limited clinical effects observed for low dose oral L-arginine[24].

Our most important finding with regards to L-arginine supplementation was that it could not rescue the impairments in HLI recovery observed in SS mice. While the ability to reduce hydrogen peroxide in our model is consistent with the antioxidant effects observed by Dasgupta et al[20], a lack of overall effect of L-arginine suggests that this is not sufficient to enhance collateralization or substantially limit inflammation. Furthermore, L-arginine had no effect on responses to HLI in control (AA) mice suggesting that the level of increased NOx achieved was not sufficient to aid recovery, even in less oxidative and inflammatory conditions. Other mechanisms may be more important in this model once ischemia is established.

Due to the increased arginase activity observed in SS mice we expected to see an increase in circulating ornithine. However, we instead observed a decrease. Interestingly, we also observed an increase in citrulline and no deficit in arginine in these mice. We therefore predicted that SS mice may have altered expression of enzymes involved in arginine synthesis and metabolism. The liver and kidney are important sites of arginine regulation and are known to be dysfunctional in SCD. In particular, impaired kidney function is associated with increased citrulline due to reduced conversion to arginine via ASS1 and ASL. Surprisingly, we did not observe any differences in ASS1 or ASL enzyme expression

in either organ. The only subtle change observed in enzyme expression was a modest increase in kidney OTC in SS mice. This may partially contribute to our findings due to enhanced conversion of ornithine to citrulline but does not seem sufficient to fully explain our results. Unfortunately, it was not feasible to measure the activity of any of these enzymes and expression levels do not give the complete picture. We speculate that although expression was not dramatically altered, there are nonetheless compensatory mechanisms occurring in SCD to limit loss of arginine and NO. This idea is supported by a study of SCD patients showing maintained citrulline and NOx levels compared to controls and increased proline, but not ornithine, in plasma[41]. Notably, we did not investigate the expression of ornithine aminotransferase (OAT) which converts ornithine to proline. Further study of these mechanisms is required to elucidate the dynamics of arginine metabolism regulated in SCD both at baseline and during acute crisis.

We report that L-arginine supplementation fails to result in any improvement in revascularization and perfusion after acute vessel injury in SCD mice, suggesting this treatment would not aid vessel recovery following ischemic events in patients. The most significant clinical findings for L-arginine treatment in patients are the substantial reductions in pain scores and opioid use in children following VOC[25,26]. However, identification of the mechanism of action for these beneficial effects was not confirmed and may not be via enhancing NO levels. Although the data are somewhat limited, mechanisms for arginine to have NO-independent analgesic actions have been identified. The arginine metabolite agmatine has been shown to enhance morphine analgesia in animal models and kytorphin is an endogenous antinociceptive synthesized from arginine and tyrosine[42]. Both of these could be increased with arginine supplementation and whether this is the primary mechanism of benefit to pain management remains to be investigated.

eNOS and NO

A protective role of eNOS and NO in collateral vessel formation and recovery of perfusion has been demonstrated in multiple studies in wildtype mice[6,7] but its effects are likely more complex in the context of SCD. NO is a highly reactive molecule that reacts rapidly with superoxide to produce the more damaging oxidant, peroxynitrite. We show that during HLI, when SCD hindlimbs are characterized by severe inflammation and oxidative stress, delivery of NO via hydrogel to the sight of injury only worsens outcomes, due at least in part to enhanced peroxynitrite generation. For this reason, we propose that strategies to enhance eNOS activity, such as BH4 co-factor treatment, or direct delivery of NO to sights of ischemia and injury, may do more harm than good to SCD patients recovering from vaso-occlusive events.

Another important observation from this study is that eNOS uncoupling does not seem to contribute to the dysfunctional response to ischemia observed in SCD mice as eNOS inhibition worsened response to HLI. This suggests that there is functional eNOS present in hindlimb that offers some level of protection and that eNOS does not contribute to the enhanced superoxide generation and inflammation observed in this model. In contrast to our results in the hindlimb, previous studies in aged (6–8 month) SCD mice implicate eNOS dysregulation in the development of pulmonary hypertension[16], and demonstrate

eNOS-dependent inflammation and platelet adhesion in cerebral venules attenuated with BH4 or superoxide dismutase (SOD) treatment[36]. These findings are consistent with our observations that L-NAME treatment limits baseline aortic inflammation in SCD mice. Taken together these studies highlight that eNOS function and the potential benefit of NO-related therapies are highly dependent on location and disease status.

SCD patients suffer from repeated bouts of ischemia and exhibit impaired wound healing responses with the common occurrence of chronic leg ulcers[43,44]. Therefore, the ability to form functional collateral vessels in response to vessel injury is important. Our previous study demonstrated that SS mice have impaired collateralization following hindlimb ischemia, associated with non-resolving neutrophilic inflammation and oxidative stress[5]. Some investigations of SCD vasculopathies have demonstrated accelerated angiogenic processes and enhanced expression of pro-angiogenic factors[45,46]. Though this may seem at odds with our observations of impaired angiogenesis following ischemia, we propose that failure to form functional collateral vessels is consistent with an enhanced, dysregulated angiogenic response, similar to those observed in Moyamoya syndrome[47], a common condition in SCD, associated with the development of dysfunctional micro vessels. Future studies could interrogate angiogenic pathways to determine whether dysregulated angiogenic signaling occurs in our model. Though predominantly used as a model of peripheral artery disease that is not commonly observed in SCD, HLI is a valuable test bed for revascularization following ischemia. Treatments that enhance collateralization and promote resolution of inflammation in this model are likely to have beneficial effects to prevent vascular complications in SCD given the central role of VOC and vessel injury to downstream organ failure and dysfunction. Furthermore, neovascularization is vitally important to wound healing that is impaired in SCD. Therefore, although not a perfect model for treatment of SCD outcomes, our results in this model strongly question the application of L-arginine and NO therapies to vascular complications in SCD. Investigation of these effects in wound healing or other relevant models of vaso-occlusive processes in SCD will be an important future direction.

Our findings also suggest that strategies to enhance eNOS activity, such as BH4 supplementation or arginase inhibition, would only be detrimental due to generation of peroxynitrite. However, there are other strategies that could be beneficial and require further investigation. Enhancement of downstream pathways that would normally be activated by NO could still be of benefit as they would bypass the potential oxidative effects of NO. For example, sGC activator BAY 54-6544 treatment was shown to limit pulmonary hypertension in SCD mice[48]. Furthermore, co-treatment with antioxidant therapies such as SOD or NAC could allow NO generation to have its beneficial effects and NAC treatment alone showed efficacy in our model[5]. More investigation is required to thoroughly tease out the mechanisms of arginine and NO regulation in SCD. The redox environment and disease context should be carefully considered for any potential therapeutic application of these treatments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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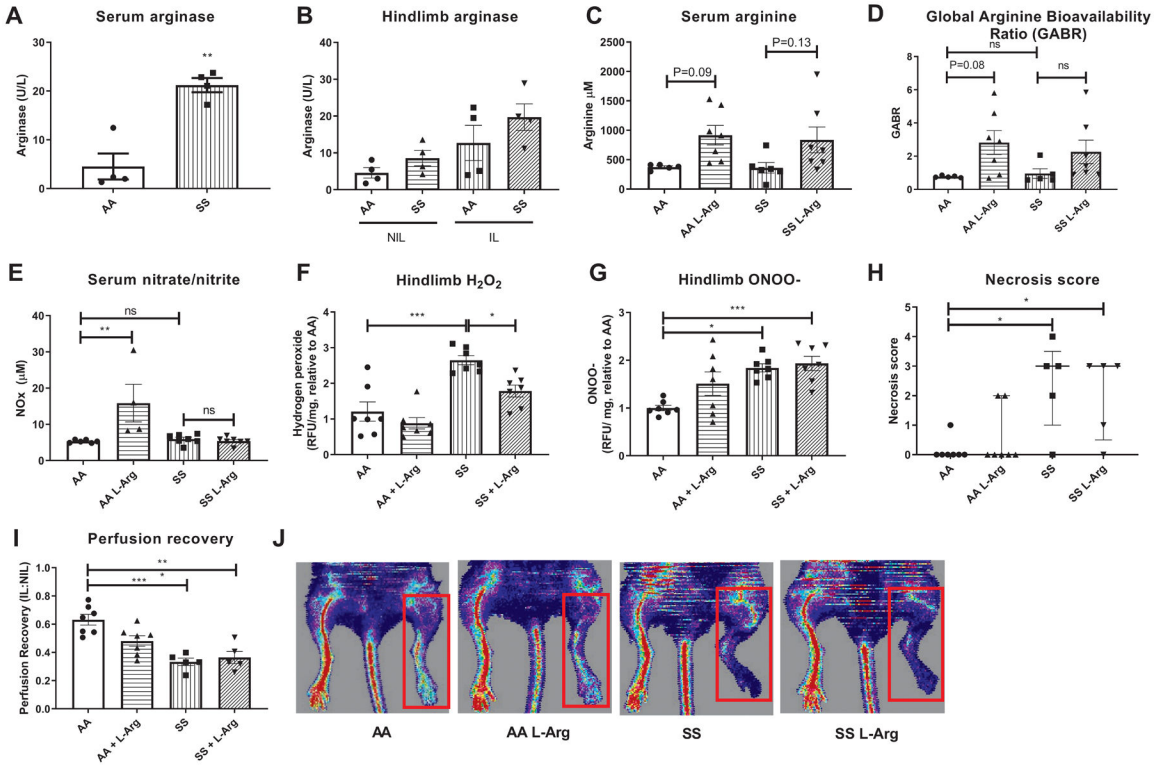

Figure 1. L-Arginine supplementation fails to improve recovery from hindlimb ischemia.

A) Arginase activity levels in serum and **B)** non-ischemic (NIL) or ischemic (IL) hindlimb 7 days following hindlimb ischemia (HLI) surgery in sickle cell mice (SS) and their wildtype littermates (AA), n=4, **P<0.01, student's unpaired t-test. **C)** Serum arginine, **D)** GABR and **E)** nitrate/nitrite (NOx) in AA and SS mice treated with L-Arginine (5% in drinking water) for 7 days, n=4–8, **P<0.01, One-way ANOVA, Sidak's post hoc test. **F)** Hydrogen peroxide (H_2O_2) and **G)** peroxynitrite (ONOO-) levels in the ischemic hindlimbs or AA and SS mice treated with L-Arginine 7 days following HLI, n=7, *P<0.05, ***P<0.001, One-way ANOVA, Sidak's post hoc test. **H)** Necrosis score and **I)** perfusion recovery (IL/NIL perfusion) in AA and SS mice treated with L-Arginine 28 days following HLI, n=4–8, *P<0.05, ***P<0.001, One-way ANOVA, Sidak's post hoc test. **J)** Representative LDPI image showing perfusion recovery in mice 28 days following HLI. IL area indicated with red box. Results expressed as mean ± SEM.

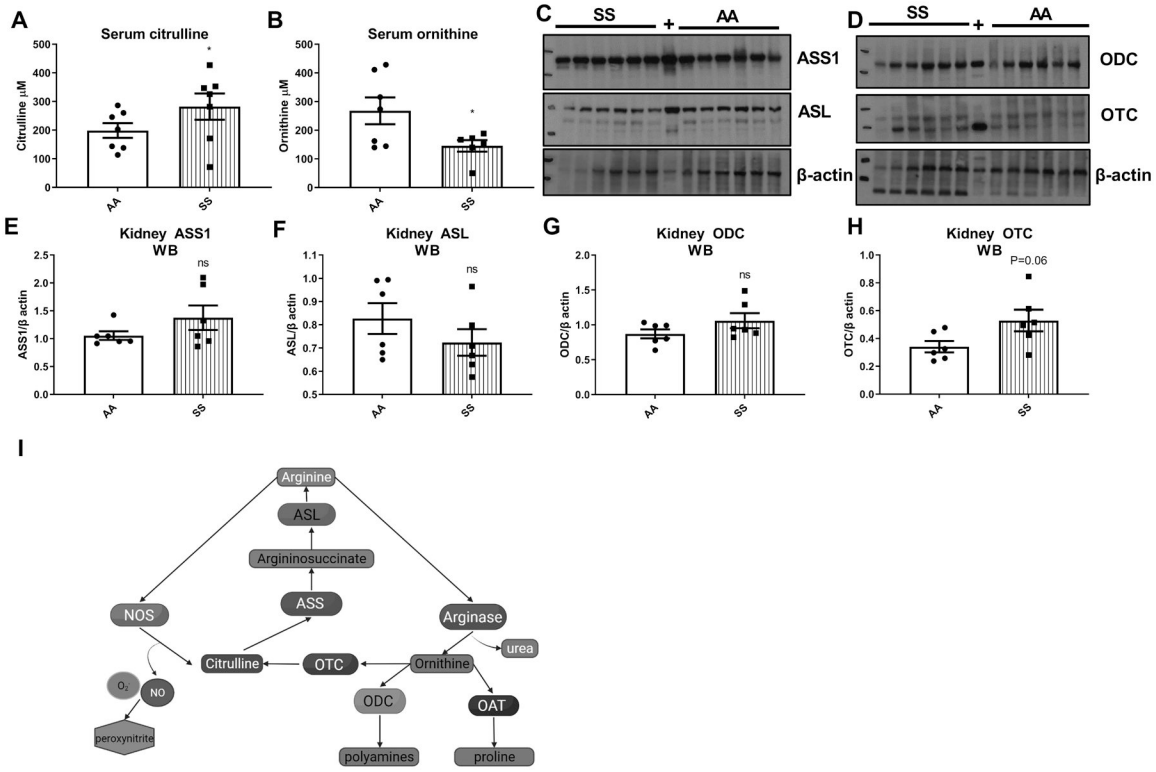

Figure 2. Analysis of arginine pathways in sickle cell mice.

A) serum citrulline and **B)** ornithine levels in sickle cell mice (SS) and their wildtype littermates (AA). **C)** Representative western blots for kidney expression of argininosuccinate synthase 1 (ASS1), argininosuccinate lyase (ASL), **D)** ornithine decarboxylase (ODC), ornthine transcarbamylase (OTC) and housekeeping protein β-actin in AA and SS mice with wildtype liver used as a positive control (+). **E)** Densitometry results for ASS1, **F)** ASL, **G)** ODC and **H)** OTC, n=6–8, *P<0.05, students unparied t-test. Results expressed as mean ± SEM. **I)** Schematic depicting enzymes and metabolites involved in arginine synthesis and cycling.

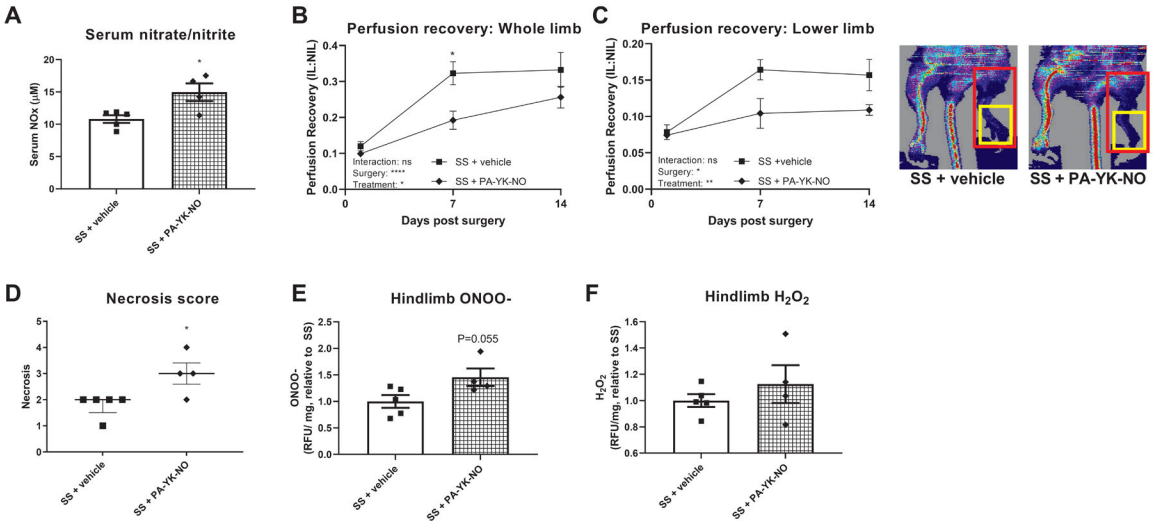

Figure 3. Nitric oxide (NO)-producing hydrogel PA-YK-NO worsens response to hindlimb ischemia in sickle cell mice.

A) Serum nitrate/nitrite (NOx) in sickle cell (SS) mice treated with vehicle or PA-YK-NO (0.9 μM NO) and subjected to hindlimb ischemia (HLI) surgery at day 14 post surgery, n=4–5, *P<0.05, student's unpaired t-test. **B)** Whole limb and **C)** lower limb perfusion recovery at day 1, day 7 and day 14 after HLI in SS mice treated with vehicle or PA-YK-NO. Representative LDPI images shown on RHS, whole limb area indicated with red box, lower limb area indicated with yellow box, n=4–5, *P<0.05, **P<0.01, ****P<0.0001, 2-way Repeated Measures ANOVA, Sidak's post hoc test. **D)** HLI Day 14 necrosis score, **E)** ischemic limb peroxynitrite (ONOO-) and **F)** ischemic limb hydrogen peroxide (H_2O_2) in SS mice treated with vehicle or PA-YK-NO, n=4–5, *P<0.05, student's unpaired t-test. Results expressed as mean ± SEM.

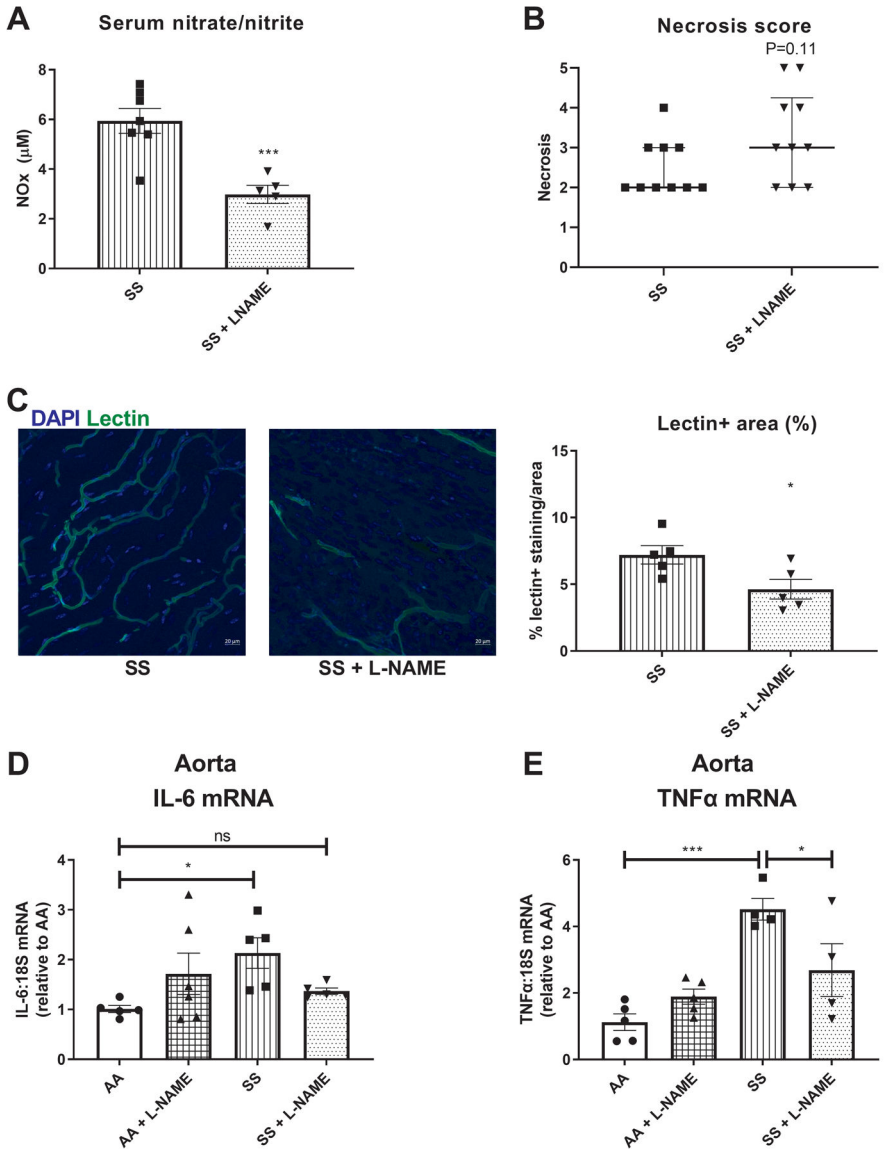

Figure 4. L-N^{G}-nitro arginine methyl ester (L-NAME) worsens response to hindlimb ischemia but protects against aortic inflammation in sickle cell mice.

A) Serum nitrate/nitrite (NOx), **B)** necrosis score and **C)** lectin positive staining (% area) in ischemic hindlimb in sickle cell (SS) mice treated with L-NAME (1 g/L in drinking water) at day 7 post hindlimb ischemia surgery, n=5 or n=10, *P<0.05, ***P<0.001, students unpaired t-test. LHS shows representative confocal microscopy image of fluorescein isothiocyanate lectin-perfused vessels showing significantly decreased vessel formation in L-NAME treated mice (scale bar= 20 μm). Aortic mRNA expression of proinflammatory cytokines **D)** interleukin 6 (IL-6) and **E)** tumour necrosis factor α (TNFα) in wildtype (AA) and sickle cell (SS) mice treated with L-NAME for 7 days, n=4–6, *P<0.05, ***P<0.001, One-way ANOVA, Sidak's post hoc test. Results expressed as mean ± SEM.