ABSTRACT

Objective: The objective of the present study was to determine whether elevated levels of S100A8 and S100A9 (S100A8/A9) alarmins contribute to ischemic limb pathology.

Methods: Gastrocnemius muscle was collected from control patients without peripheral arterial disease (PAD; n = 14) and patients with chronic limb threatening limb ischemia (CLTI; n = 14). Mitochondrial function was assessed in permeabilized muscle fibers, and RNA and protein analyses were used to quantify the S100A8/A9 levels. Additionally, a mouse model of hindlimb ischemia with and without exogenous delivery of S100A8/A9 was used.

Results: Compared with the non-PAD control muscles, CLTI muscles displayed significant increases in the abundance of S100A8 and S100A9 at both mRNA and protein levels (P < .01). The CLTI muscles also displayed significant impairment in mitochondrial oxidative phosphorylation and increased mitochondrial hydrogen peroxide production compared with the non-PAD controls. The S100A8/A9 levels correlated significantly with the degree of muscle mitochondrial dysfunction (P < .05 for all). C57BL6J mice treated with recombinant S100A8/A9 displayed impaired perfusion recovery and muscle mitochondrial impairment compared with the placebo-treated mice after hindlimb ischemia surgery. These mitochondrial deficits observed after S100A8/A9 treatment were confirmed in the muscle cell culture system under normoxic conditions.

Conclusions: The S100A8/A9 levels were increased in CLTI limb muscle specimens compared with the non-PAD control muscle specimens, and the level of accumulation was associated with muscle mitochondrial impairment. Elevated S100A8/A9 levels in mice subjected to hindlimb ischemia impaired perfusion recovery and mitochondrial function. Together, these findings suggest that the inflammatory mediators S100A8/A9 might be directly involved in ischemic limb pathology.

Clinical Relevance: Despite improvements in the surgical management of chronic limb threatening limb ischemia (CLTI), the rates of major adverse limb events have remained high. Skeletal muscle has emerged as a strong predictor of outcomes in peripheral arterial disease (PAD)/CLTI; however, a complete understanding of muscle pathology in CLTI is lacking. This study identified elevated S100A8 and S100A9 alarmin proteins as a characteristic of CLTI muscle specimens and that the S100A8/A9 levels are associated with the degree of mitochondrial impairment in patient limb muscle specimens. Using a mouse model of PAD, treatment with S100A8/A9 exacerbated ischemic limb pathology, including impaired perfusion recovery and muscle mitochondrial impairment. Taken together, these findings connect the inflammatory milieu in the CLTI limb to exacerbated limb muscle outcomes via mitochondrial alterations.

Keywords: Inflammation; Mitochondria; Peripheral artery disease; Vascular disease
about the underlying biology by which this inflammatory state might contribute to ischemic limb pathology.

Recent studies have implicated S100A8 and S100A9 (S100A8/A9), proinflammatory calcium binding proteins from the S100 family known as alarmins, as regulators of ischemic pathology in the myocardium. S100A8/A9 are expressed abundantly in immune cells (neutrophils and macrophages) that infiltrate tissues after ischemic events, environmental triggers, and/or cellular damage and can be released at these local sites from these phagocytes. The S100 family members S100A8 and S100A9 are the best studied and are found primarily as heterodimers in vivo (excellent reviews on S100 proteins have been provided by Sreejit et al). Within cells, these alarmins participate in cytoskeletal rearrangements and alter the metabolism but are actively released from immune cells during inflammation to contribute to leukocyte recruitment and subsequent cytokine secretion. S100A8/A9 are also known ligands for toll-like receptor 4 and the receptor for advanced glycation end products, which have been implicated in the pathologic effects of these alarmins. In patients, the plasma levels of S100A8/A9 correlate with circulating neutrophil counts and have been associated with cardiovascular disease risk. Mechanistically, S100A8/A9 were recently shown to cause cardiomycocyte mitochondrial dysfunction (inhibition of complex I of the electron transport system) and to increase cell death after myocardial infarction in mice. Another group recently reported that treatment with a selective S100A9 blocker, ABR-238901, significantly improved cardiac function in mice after myocardial infarction.

Information regarding the relevance of these alarmins to PAD has begun to emerge in the literature. A recent study from Spain reported that elevated S100A9 levels in extracellular vesicles obtained from serum distinguished patients with intermittent claudication from controls and patients with CLTI from those with intermittent claudication. Furthermore, they reported that S100A9 levels had prognostic value in relation to major adverse cardiovascular events and amputation outcomes. Of relevance to human patients with PAD, a recent study of diabetic mice demonstrated that pharmacologic blockage of S100A8/A9 reduced atherogenesis. In the murine hindlimb ischemia model, Ganta et al demonstrated that VEGFR1 (vascular endothelial growth factor receptor 1) inhibition by the antiangiogenic isofrom VEGF165b in macrophages resulted in S100A8/A9-mediated calcium influx, inducing an M1-like phenotype and impairing ischemic tissue regeneration. Combined with the abundance of data from myocardial ischemia, these findings suggest that S100A8/A9 might play a significant role in PAD pathobiology. Thus, we hypothesized that elevated S100A8/A9 levels would contribute to exacerbated ischemic limb pathology. Therefore, we first assessed the S100A8/A9 levels in CLTI and non-PAD limb muscle specimens. Next, we treated C57BL6J mice with exogenous S100A8/A9 to determine whether increasing the levels of these alarmins would exacerbate ischemic limb pathology.

**METHODS**

Human study populations and specimen collection. Gastrocnemius muscle specimens were collected from 14 older adult non-PAD controls and 14 CLTI patients undergoing limb amputation (5 above the knee and 5 below the knee) or surgical intervention (angioplasty for 2 and bypass for 2). Of the CLTI amputation specimens, four were collected from patients with nonhealing ulcers or gangrene. Seven patients had undergone previous surgical interventions (three angioplasty, four bypass), and one sample was obtained from a CLTI patient with nonreconstructable disease. The specimen was collected below the knee or surgical intervention (angioplasty for 2). Of the CLTI amputation specimens, 14 older adult non-PAD controls and 14 CLTI patients undergoing limb amputation (5 above the knee and 5 below the knee) or surgical intervention (angioplasty for 2 and bypass for 2). The operating room for the CLTI patients or via percutaneous biopsies, his-
Tandem mass tag-labeled proteomics analysis of human skeletal muscle. A portion of the snap frozen muscle specimen was used for isobaric tandem mass tag-labeled proteomic analysis. We described the tissue processing, mass spectrometry analysis, and bioinformatics for these samples in our previous study. Protein abundance for S100A8 and S100A9 from these proteomics analyses was converted to the Log2 space for group comparisons. For each comparison, protein abundance was analyzed for the group average, standard deviation, two-tailed Student’s t-test (equal variance), and a Benjamini-Hochberg false discovery rate-corrected adjusted P-value. All raw proteomics data are available online using accession number PXD021849 (Proteome Xchange Consortium; available at: http://www.proteomexchange.org/) or JPST000852 (Japan Proteome Standard Repository; available at: https://repository.jpostdb.org/).

RNA analysis of human skeletal muscle. A portion of snap frozen muscle was used for RNA isolation using Trizol reagent to homogenize the specimen and the Directzol RNA miniprep kit (model no. R2072; Zymo Research, Irvine, CA) in accordance with the manufacturer’s instructions. Following RNA isolation, cDNA was synthesized using 200 ng of RNA with the qScript cDNA synthesis kit (model no. 95047-500; Quantabio, Beverly, MA). Real-time polymerase chain reaction was performed using QuantStudio 3 (Applied Biosystems, Waltham, MA) with the TaqMan fast advanced master mix (Applied Biosystems) and the following TaqMan probes: Hs00374264_g1 (S100A8), Hs00610058_m1 (S100A9), and Hs03003631_g1 (18S). Gene expression was normalized to the internal loading control 18S, and relative changes in the mRNA levels were quantified using the ΔΔCT method.

Preparation of permeabilized human muscle fibers. A portion of the muscle biopsy specimen was dissected and immediately placed in ice-cold buffer X (50 mM K-MES, 7.23 mM K2EGTA, 2.77 mM CaK2EGTA, 20 mM imidazole, 20 mM taurine, 5.7 mM adenosine triphosphate [ATP], 14.3 mM phosphocreatine, and 6.56 mM MgCl2·6H2O; pH 7.1) for preparation of permeabilized fiber bundles, as previously described by our group. Before loading the fiber bundles for experimentation, they were gently blotted on a Kimwipe (Kimberly-Clark Professional Kimtech Science; Thermo Fisher Scientific, Waltham, MA) for exactly 5 seconds, and a wet weight was obtained using a Mettler Toledo MX5 microbalance (Mettler Toledo, Columbus, OH).

Mitochondrial respiration measurements in human muscle fibers. High-resolution oxygen consumption measurements were conducted at 37°C in buffer Z (105 mM K-MES, 30 mM KCl, 1 mM EGTA, 10 mM K2HPO4, 5 mM MgCl2·6H2O, 0.5 mg/mL bovine serum albumin [BSA]; pH 7.1), supplemented with creatine monohydrate (5 mM), using an O2K Oxigraph (Oroboros Instruments, Innsbruck, Austria). To assess mitochondrial function under physiologically relevant conditions, we used a novel creatine kinase (CK) clamp system to set the level of cellular energy demand to which the fiber bundles were exposed. First, the bundles were energized with either carbohydrate (5 mM pyruvate and 2.5 mM malate) or fatty acid (0.2 mM octanoylcarnitine and 2.5 mM malate), and measurements of state 2 oxygen consumption were collected. Next, the CK clamp was added, which included 20 U/mL of creatine kinase, 5 mM ATP, and 1 mM phosphocreatine (PCr) to mimic near maximal exercise conditions. PCr was subsequently added in a stepwise fashion to reduce the cellular energy demand to resting conditions. The slope of the relationship between the cellular energy demand (ΔGATP) and oxygen consumption (ΔO2) was calculated. The rate of respiration was expressed as pmol/s/mg fiber wet weight. All respiration measurements were conducted at 37°C and a working range [O2] of ~350 to 200 μM.

Mitochondrial hydrogen peroxide emission. Mitochondrial hydrogen peroxide (H2O2) emission was measured in the bundles fluorometrically at 37°C via the Amplex UltraRed (10 μM)/horseradish peroxidase (3 U/mL) detection system (Thermo Fisher Scientific; excitation/emission 565/600) using a Horiba Quantamaster 400 (Horiba, Kyoto, Japan), as previously described. This analysis was performed using the identical substrate conditions described in the previous sections. The fluorescence units were converted to pmols of H2O2 using a standard curve.

Mice. C57BL/6J male mice (stock no. 000664) were obtained from The Jackson Laboratory (Bar Harbor, ME) and used at 12 weeks of age (n = 20 total). All the mice were housed in a temperature-controlled (22°C) and light-controlled (12-hour light/12-hour dark) room and fed a standard chow diet with free access to food and water. All animal experiments adhered to the Guide for the Care and Use of Laboratory Animals from the Institute for Laboratory Animal Research (National Research Council.
Hindlimb ischemia surgery. Unilateral hindlimb ischemia was induced by anesthetizing the mice with an intraperitoneal injection of ketamine (90 mg/kg) and xylazine (10 mg/kg) and surgically placing silk ligatures on the femoral artery from its origin just distal to the inguinal ligament and immediately distal to the proximal caudal femoral artery. Superficial limb necrosis was not observed in any of the mice. At 7 days after induction of hindlimb ischemia, the mice were anesthetized with ketamine and xylazine for muscle tissue procurement and the experiments as described in the next sections.

Laser Doppler limb perfusion measurements. Limb perfusion was measured using a laser Doppler flowmeter (moorVMS-LDF; Moor Instruments, Wilmington, DE) before surgery, immediately after surgery, and just before sacrifice (7 days after surgery) under ketamine and xylazine anesthesia. In brief, the hindlimbs were shaved to remove the hair, and the laser Doppler probe was carefully placed against the skin of the lateral head of the gastrocnemius muscle and the bottom of the paw. Data were collected continuously for 60 seconds, and the average perfusion rate was calculated. Perfusion recovery in the ischemic limbs was calculated as a percentage of the average perfusion rate in the nonischemic control limb, as previously described.

Skeletal muscle morphology. Skeletal muscle morphology was assessed using standard light microscopy. Transverse sections, 10-μm thick, from the tibialis anterior muscle were cut using a cryotome (model no. CM3050S; Leica Biosystems, Baden-Wurttemberg, Germany) and collected on slides for staining. For morphological analyses, the standard methods for hematoxylin and eosin histologic staining were used, and images were obtained at 20× magnification using automated image capture and/or tiling to image the entire muscle section using an Evos FL2 Auto microscope (Thermo Fisher Scientific). All image analyses were conducted by a blinded investigator using ImageJ (National Institutes of Health, Bethesda, MD). Injured or regenerating myofibers were quantified by manually counting the fibers with centralized nuclei from the entire section of the muscle (imaged and tiled together).

Immunofluorescence microscopy. The skeletal myofiber cross-sectional area and capillary density were assessed in mouse skeletal muscles using immunofluorescence microscopy, as previously described. We cut 10-μm-thick transverse sections from the tibialis anterior muscle. The sections were fixed with 4% paraformaldehyde, subsequently blocked for 1 hour at room temperature with phosphate-buffered saline (PBS) supplemented with 5% goat serum and 1% BSA, and incubated overnight at 4°C with a primary antibody for laminin (1:100; catalog no. L9393, Sigma-Aldrich, St Louis, MO) to label the myofibers membrane. Following washes with PBS, the sections were incubated with Alexa-Fluor secondary antibodies (1:250; Thermo Scientific Fisher) and Dylight594-conjugated Griffonia simplicifolia I isolecitin B4 (DL-1207; Vector Laboratories, Burlingame, CA) to label endothelial cells (ie, capillaries). Coverslips were mounted with Vectashield HardSet mounting medium containing DAPI (4',6-diamidino-2-phenylindole; H-1500; Vector Laboratories). Images were obtained at 20× magnification using an Evos FL2 Auto microscope (Thermo Fisher Scientific). All image analyses were performed using MuscleJ, an automated image analysis program in Fiji. The total capillary counts were collected from the tiled images of the entire tibialis anterior muscle of each mouse.

Assessment of muscle contractile function. Muscle contractile function was assessed ex vivo in the extensor digitorum longus muscle, as previously described. We measured the isometric forces at stimulation frequencies of 1, 20, 40, 60, 100, and 150 Hz using a biphasic high-power stimulator (701C; Aurora Scientific, Aurora, ON, Canada) delivered with current of 600 mA, pulse duration of 0.25 ms, and train duration of 500 ms, with each stimulation train separated by 1-minute intervals of rest. The isometric forces were normalized by the cross-sectional area, which was estimated by measuring the muscle weight and length at the optimal muscle length. The muscle weight was divided by the length multiplied by 1.06 g/cm², the density of mammalian skeletal muscle.

Mouse muscle mitochondrial isolation and respiratory function. Hindlimb muscle mitochondrial respiratory function was tested using the gastrocnemius muscle in the hindlimb and was isolated as previously reported. Using the CK clamp system described, respiratory function was assessed at 37°C in buffer Z (in mmol/L) supplemented with creatine monohydrate (5 mM), using the O2K Oxymograph (Oroboros Instruments). Isolated mitochondria were energized with pyruvate and malate (5 mM and 2.5 mM, respectively) to obtain state 2 respiration, followed by addition of the CK clamp and the addition of PCr to titrate the energy demand from a mimicked high exercise level to resting levels. The slope of the relationship between the cellular energy demand (ΔΔATP) and oxygen consumption (JO2) was calculated. The rate of respiration was expressed as pmol/oxygen consumption (ΔΔATP)/mg of mitochondria. All respiration measurements were conducted at 37°C and a working range [O2] of ∼200 μM. H2O2 production was assessed using pyruvate and malate and identical energy demands as performed using

Washington, DC, 1996 and any updates). The institutional animal care and use committee of the University of Florida approved all the procedures.
the O2K Oxygraph (Oroboros Instruments) via the Amplex UltraRed (10 μM)/horseradish peroxidase (3 U/mL) detection system (Thermo Fisher Scientific), as previously described.21,24

**Muscle cell culture.** C2C12 muscle cells were obtained from American Type Culture Collection (myoblast cell line, CRL-1772) and cultured in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in standard conditions (37°C; 5% carbon dioxide). Myoblast differentiation was initiated by serum withdrawal using Dulbecco’s modified Eagle medium supplemented with 2% heat-inactivated horse serum and 1% penicillin/streptomycin. All culture experiments were performed in three biologically independent lots.

**Myotube atrophy.** Myoblasts were differentiated for 7 days before treatment with recombinant S100A8/A9 (rS100A8/A9: 1000 ng/mL) or placebo (PBS supplemented with 0.1% BSA). After 24 hours of treatment with rS100A8/A9 or placebo, the cells were washed with PBS, fixed with 100% methanol for 10 minutes, left to air dry for 10 minutes, and incubated with primary antibody against sarcomeric myosin (MYH1E antibody was deposited to the Developmental Studies Hybridoma Bank by Fischman DA; DSHB Hybridoma Product) at 1:25 in blocking solution (AlexaFluor594, mouse IgG2b; Thermo Fisher Scientific) for 1 hour at 37°C. The cells were then washed three times in PBS, followed by incubation with 1250 secondary antibody (AlexaFluor594, mouse IgG2b, Thermo Fisher Scientific) for 1 hour at 37°C. The myotubes were then washed three times in PBS and imaged using automated capture routines with an Evos FL Auto 2 inverted fluorescence microscope (Thermo Fisher Scientific). Each well was imaged using automation to cover 60% of the well starting from the center. The myotube area (MYH1E antibody-positive area) was analyzed using custom written routines in CellProfiler (Broad Institute of MIT and Harvard, Cambridge, MA). All processing procedures were performed uniformly over the entire set of images using batch processing modes to remove any human input.

**Myotube mitochondrial function.** C2C12 myoblasts were differentiated for 7 days before treatment with rS100A8/A9 (1000 ng/mL) or placebo (PBS supplemented with 0.1% BSA) for 24 hours. Following treatment, the myotubes were washed one time with PBS, trypsinized and collected, and centrifuged at 800g for 5 minutes. The pelleted cells were then resuspended in buffer Z supplemented with 5 mM creatine. Respiratory function was assessed via the O2K Oxygraph (Oroboros Instruments) at 37°C and a working range of O2 of ~350 to 200 μM. First, intact cell respiration was acquired, followed by the addition of digitonin (10 μg/mL) to permeabilize the cells. Pyruvate (5 mM) and malate (2.5 mM) were added to induce state 2 respiration, followed by implementation of the CK clamp and sequential titrations of PCr to determine the respiratory function at each energy demand imposed (ΔGATP, 54.12-63.8 kJ/mol), and mitochondrial oxidative phosphorylation conductance was determined as the slope of the JO2 vs ΔGATP relationship. As described, H2O2 was also assessed in myotubes using the Amplex UltraRed (10 μM)/horseradish peroxidase (3 U/mL) detection system (Thermo Fisher Scientific).

**Statistical analysis.** The data are presented as the mean ± standard deviation. The normality of all the data was assessed using the Shapiro-Wilk test. Data that were not normally distributed were analyzed using a Kruskal-Wallis test. Comparisons across the two groups were performed using one-way analysis of variance with Tukey’s post hoc multiple comparisons when pairwise comparisons were appropriate or a two-tailed Student’s t test otherwise. A χ2 test was used analyze the differences in population proportions for the relevant clinical characteristics. All statistical analyses were performed using Prism, version 8.0 (GraphPad, San Diego, CA), or VassarStats (available at: http://vassarstats.net). In all cases, P < .05 was considered statistically significant.

**RESULTS**

**S100A8 and S100A9 are elevated in CLTI limb muscle specimens.** In the present study, limb muscle specimens were collected from non-PAD control participants and CLTI patients undergoing limb amputation or surgical intervention. The physical and clinical characteristics of the study participants are listed in the Table. The CLTI and non-PAD control groups were relatively well-matched for age. However, the control group had fewer comorbid conditions and less medication usage. The CLTI patient comorbidities included hypertension (100%), hyperlipidemia (100%), diabetes mellitus (64%), coronary artery disease (64%), chronic obstructive pulmonary disease (21%), and renal disease (21%). Most (79%) of the CLTI patients were either former or active smokers. In contrast, only 36% of the non-PAD control group were either former or active smokers.

We previously reported the results of a proteomic analysis of CLTI limb muscle (accession no. PXD021849 [Proteome Xchange Consortium]; or accession no. JPST000852 [Japanese Proteome Standard Repository]).25 Although not the focus of our previous study, the protein abundance of S100A8 and S100A9 was significantly higher in the CLTI muscle than in the non-PAD control muscle (Fig 1A). These results were further confirmed at the mRNA level using real-time polymerase chain reaction. The CLTI limb muscle displayed greater mRNA levels of both S100A8 and S100A9 compared with the non-PAD control muscles (Fig 1B). The patients with the highest protein or mRNA abundance of S100A8/A9 had all had a diagnosis of Rutherford class 5 and 6. In contrast, some patients with Rutherford class 4 had displayed expression levels
near those of the control group. A representative image of hematoxylin and eosin–stained muscle sections confirmed the presence of immune and inflammatory cell infiltrates and evidence of myopathy, including fibers with centralized nuclei, an irregular fiber shape, and necrotic myofibers (Fig 1, C).

S100A8 and S100A9 protein levels correlate with mitochondrial impairment in CLTI. Consistent with our previous reports,\textsuperscript{24,25} we found that CLTI patients displayed significant impairment in mitochondrial oxidative phosphorylation. In the present study, we used a novel CK clamp system to test the mitochondrial oxidative phosphorylation function across a range of physiologic relevant energy demands to mimic a stress test that ranges from rest to exercise. Whether energized with saturating concentrations of carbohydrates (pyruvate and malate) or fatty acids (octanoylcarnitine and malate), the CLTI muscle bundles displayed significantly lower rates of oxygen consumption compared with the non-PAD control muscle bundles (Fig 2, A and C). Quantification of the slope of these graphs provided an index of oxidative phosphorylation function (termed conductance), which was significantly lower in the CLTI muscle with both carbohydrates (Fig 2, B) and fatty acids (Fig 2, D). Correlational analyses revealed a significant inverse relationship between the protein abundance of S100A8 or S100A9 and oxidative phosphorylation function (Fig 2, E), a finding consistent with that from a previous study reporting that these alarmins impair mitochondrial function in the heart muscle.\textsuperscript{18}

Next, we used the same mitochondrial stress testing procedure to examine mitochondrial H₂O₂ production, a well-known reactive oxygen species. Using the Amplex UltraRed fluorescent dye, the CLTI muscle bundles displayed significantly greater levels of H₂O₂ production compared with the non-PAD control muscle bundles for both carbohydrate (Fig 3, A) and fatty acid (Fig 3, B) substrates. Correlational analyses demonstrated a significant positive correlation between the protein abundance of S100A8 or S100A9 and the level of H₂O₂ production at a resting energy demand (ΔGATP ≈ 63.90 kJ/mol) regardless of the substrate (Fig 3, C). Taken together, these findings suggest that the level of S100A8 and/or S100A9 in the ischemic limb could be a contributing factor to the degree of mitochondrial pathology in CLTI.

Table. Physical and clinical characteristics

| Characteristic                                      | Control (n = 14) | CLTI (n = 14) | P value |
|-----------------------------------------------------|------------------|--------------|---------|
| Age, years                                          | 71.9 ± 6.3       | 68.5 ± 7.2   | .299    |
| Female sex                                          | 4 (29)           | 1 (7)        | .044    |
| Overweight/obese (BMI ≥ 25 kg/m²)                   | 9 (64)           | 9 (64)       | 1.000   |
| ABI                                                 | 1.09 ± 0.11      | 0.53 ± 0.25\(^b\) | \(<.001^a\) |
| Rutherford classification                           |                  |              |         |
| 0                                                   | 14 (100)         | 0 (0)        | \(<.001\) |
| 4                                                   | 0 (0)            | 6 (43)       | .624    |
| 5                                                   | 0 (0)            | 6 (40)       | .646    |
| 6                                                   | 0 (0)            | 2 (14)       | .454    |
| Medication                                          |                  |              |         |
| Diabetes mellitus, type 1 or 2                      | 6 (43)           | 9 (64)       | .108    |
| Hypertension                                        | 8 (57)           | 14 (100)     | .010    |
| Hyperlipidemia                                      | 6 (43)           | 14 (100)     | .006    |
| Coronary artery disease                             | 2 (14)           | 9 (64)       | .004    |
| Renal disease                                       | 0 (0)            | 3 (21)       | .183    |
| COPD                                                | 1 (7)            | 3 (21)       | .283    |
| Tobacco use                                         | 5 (36)           | 11 (79)      | .001    |
| Former smoker                                       | 3 (21)           | 8 (57)       | .006    |
| Current smoker                                      | 2 (14)           | 3 (21)       | .335    |
| Medication                                          |                  |              |         |
| Aspirin                                             | 5 (36)           | 10 (71)      | .007    |
| Statin                                              | 5 (36)           | 14 (100)     | \(<.001\) |
| ACE inhibitor                                       | 5 (50)           | 6 (60)       | .784    |
| Cilostazol                                          | 0 (0)            | 6 (43)       | \(<.001\) |

ABI, Ankle brachial index. ACE, angiotensin-converting enzyme. BMI, body mass index. CLTI, chronic limb threatening ischemia. COPD, chronic obstructive pulmonary disease. Data presented as mean ± standard deviation or number (%). Boldface P values represent statistical significance.

\(^a\)Chi-square test (performed to determine differences in population proportions).

\(^b\)Four patients with CLTI had noncompressible vessels, precluding ABI measurement.

Treatment with S100A8/A9 impairs perfusion recovery by not muscle contraction in C57BL6J mice. To examine a potential causal relationship between ischemic limb pathology and S100A8/A9 levels, we used the widely accepted hindlimb ischemia model of PAD in mice. C57BL6J mice received an intraperitoneal injection of recombinant S100A8/A9 (50 μg/kg body weight) or placebo (0.1% BSA in sterile saline) the day before surgery and subsequently every other day until sacrifice (Fig 4, A). The mice treated with rS100A8/A9 showed significantly impaired perfusion recovery in the gastrocnemius muscle (\(P < .05\)) and a nonsignificant decrease in paw perfusion (\(P = .09\); Fig 4, B). The total capillary count in the nonischemic or ischemic extensor digitorum longus muscles was not different between groups (Fig 4, C). Histologic analysis of the tibialis anterior muscle showed a similar degree of muscle injury and regeneration, evidenced by equal numbers of myofibers with centralized nuclei and myofiber sizes (Fig 4, D-F). In line with the histologic evidence, the force-producing capacity of the muscle was not altered by rS100A8/A9 treatment in either the control or ischemic extensor digitorum longus muscles (Fig 4, C).
Treatment with S100A8/A9 impairs muscle mitochondrial function in C57BL6J mice. Because the abundance of S100A8 and S100A9 in CLTI patient muscles was associated with the magnitude of mitochondrial impairment, we used this mitochondrial assay to determine whether treatment with rS100A8/A9 impaired mitochondrial function in mice. We found that mice treated with rS100A8/A9 displayed reduced mitochondrial oxygen consumption (JO2) across a range of energy demands in both nonischemic (Fig 5, A) and ischemic (Fig 5, B) gastrocnemius muscle mitochondria. However, in contrast to the strong association between the abundance of S100A8/A9 and mitochondrial H2O2 production in CLTI muscles, mice treated with rS100A8/A9 did not display greater production rates of H2O2 in either limb (Fig 5, C and D).

Treatment with S100A8/A9 causes atrophy and mitochondrial impairment in cultured myotubes. To isolate the effects of rS100A8/A9 from the effects of impaired muscle perfusion (Fig 4, B) observed in the mouse experiment, we used a cultured myotube system in which matured myotubes were treated with rS100A8/A9 in normoxic culture conditions. C2C12 myotubes (day 7 of differentiation) were treated with 1000 ng/mL of rS100A8/A9 by supplementing the culture medium for 24 hours. rS100A8/A9 treatment resulted in significant myotube atrophy (Fig 6, A and B) compared with vehicle treated (PBS plus 0.1% BSA) myotubes. Next, we used the same physiologic mitochondrial phenotyping system to analyze a range of steady state energy demands (Fig 6, C). These analyses of mitochondrial oxidative phosphorylation confirmed that rS100A8/A9 treatment impaired mitochondrial oxidative phosphorylation (Fig 6, D) but did not alter mitochondrial H2O2 production (Fig 6, E). These findings were consistent with the observed changes in the mice treated with rS100A8/A9 and confirmed that these alarmins induce mitochondrial impairment in muscle cells.

DISCUSSION

Inflammation is common in patients with PAD and has been shown to be intimately linked to both the development and the progression of the disease.14,5,40–43 PAD risk factors, including tobacco smoking and diabetes, both independently increase inflammation.45 Previous studies have identified several inflammatory molecules, including C-reactive protein, interleukin-6, and soluble intracellular adhesion molecule-1, that have been linked with both the development of PAD and its associated health outcomes.45,46,47 Although inflammation has generally been regarded as a contributing factor to the pathobiology of cardiovascular disease, including PAD, a recent study failed to replicate the prognostic abilities of C-reactive protein in patients with PAD.48 Thus,
much remains to be discovered regarding how inflammatory mediators affect PAD pathology. Careful examination of a recently curated proteomics dataset from CLTI limb muscle indicated that S100A8/A9 proteins were significantly elevated in CLTI muscle compared with non-PAD control participants (Fig 1, A).25 This finding was confirmed using mRNA analysis (Fig 1, B). S100A8/A9 are calcium binding proteins that belong to the S100 family. They are often found as heterodimers owing to the increased structural stability compared with homodimers. They are constitutively expressed in immune cells such as neutrophils and monocytes but will be released by these cells during inflammatory states.6,49 The observation that S100A8/A9 abundance was elevated in the CLTI limb peaked our interest because recent studies had demonstrated that these calcium binding proteins might play a causal role in pathology after myocardial infarction.9 S100A8/A9 protein levels increased with myocardial ischemia and were detectable in serum before other biochemical indicators of myocardial necrosis.29 In a rodent model of postischemic heart failure, a study reported that S100A8/A9 mRNA and proteins levels increased rapidly following induction of ischemia and that treatment with recombinant S100A8/A9 impaired cardiac performance after ischemia and reperfusion.10 Preclinical studies have shown that blockage of S100A8/A9 can improve cardiac pathology after myocardial ischemia.19,49 Mechanistically, a recent study indicated that S100A8/A9 proteins are linked to myocardial ischemic pathology via inhibition of mitochondrial metabolism in cardiomyocytes.18 Our group, and others, reported that aberrant mitochondrial metabolism is a common feature of limb muscle specimens from PAD/CLTI patients.24,50 Using permeabilized muscle fibers and high respirometry, such as in our previous study,24 we assessed muscle mitochondrial metabolism using a novel energy clamp system that assesses mitochondrial oxygen consumption across a range of physiologic energy demands (similar to a stress test).28 Consistent with previous findings, CLTI muscle displayed a significant impairment in mitochondrial oxidative phosphorylation (Fig 2, A-D). The degree of mitochondrial impairment correlated strongly with the S100A8 and S100A9 protein levels (Fig 2, E).
Mitochondrial H$_2$O$_2$ production was also greater in the CLTI muscle than in the non-PAD control muscle (Fig 3, A and B). Also, the level of H$_2$O$_2$ production at a resting level of energy demand correlated with the S100A8/A9 protein levels (Fig 3, C).

To determine whether elevated S100A8/A9 levels could regulate ischemic limb pathology, we used the established hindlimb ischemia model in C57BL6 mice—a genetic strain known to be relatively resistant to CLTI symptoms.$^{31,33,37,38,56-58}$ Consistent with worsening ischemic myocardial function,$^{10}$ the mice given intra-peritoneal injections of recombinant S100A8/A9 displayed impaired perfusion recovery after induction of hindlimb ischemia (Fig 4, B). Our finding of impaired limb perfusion recovery in the S100A8/A9-treated mice subjected to hindlimb ischemia was supported in part by a previous study. Ganta et al.$^{22}$ reported that the anti-angiogenic isoform VEGF$_{165b}$, which is elevated in PAD limb muscle, inhibits VEGFR1 in macrophages, causing the S100A8/A9-mediated M1-like phenotype and impairing angiogenesis and perfusion recovery in ischemic muscle. The findings from the present study have extended the increasing body of literature demonstrating that macrophage maturation plays a critical role in arteriogenesis and angiogenesis—a process highly relevant to the CLTI population.$^{59-61}$ These findings have shown that it is plausible that the injected rS100A8/A9 could have negatively affected macrophages, contributing to the observed impairment in perfusion recovery (Fig 3, B), although flow cytometry-based assessments of macrophage maturation and polarization were not performed in the present study.

Given the striking correlation between the abundance of S100A8/A9 and mitochondrial impairment in CLTI patient muscle specimens, we also assessed the limb muscle mitochondrial function in mice treated with either placebo or rS100A8/A9. Consistent with the findings in cardiomyocytes, the mice treated with rS100A8/A9 exhibited impairments in muscle mitochondrial oxidative phosphorylation in both nonischemic and ischemic
This finding of S100A8/A9-mediated mitochondrial respiratory impairment was also confirmed in a cultured myotube system in which oxygen availability was not limiting (Fig 6). Taken together, these findings have confirmed that, similar to cardiomyocytes, S100A8/A9 can directly impair skeletal muscle mitochondrial function. Although rS100A8/A9 treatment in mice impaired mitochondrial respiration, it did not increase mitochondrial H$_2$O$_2$ production (Fig 5). The rate of H$_2$O$_2$ production in these experiments was dependent on the balance between the rate of production and the rate of scavenging by the numerous antioxidant systems resident in mitochondria. Thus, a decrease in the rate of respiration is not a direct predictor of H$_2$O$_2$ or superoxide production. Moreover, it is important to recognize that within the assay conditions used, mitochondria were exposed to physiologically relevant energy demands in which very few electrons flowing through the electron transport system will leak prematurely to or from superoxide or H$_2$O$_2$.  

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**Fig 4.** Treatment with recombinant S100A8 and S100A9 (rS100A8/A9) impaired limb perfusion recovery but not muscle force production in mice subjected to hindlimb ischemia. 

A, Schematic of the mouse model of hindlimb ischemia with either placebo or rS100A8/A9 treatment. B, Laser Doppler perfusion recovery in the paw and gastrocnemius muscle. C, Capillary density in the tibialis anterior muscle of the nonischemic and ischemic limb of mice. D, Representative hematoxylin and eosin–stained tibialis anterior muscles indicating mild injury. E, Quantification of the number of myofibers with centralized nuclei (indicating muscle injury and/or regeneration). F, Quantification of the myofiber cross-sectional area in the nonischemic and ischemic tibialis anterior muscles. G, Muscle force production measured ex vivo in the extensor digitorum longus muscles (n = 10/group). *P < .05, repeated measures analysis of variance.
Despite decreasing limb perfusion recovery and mitochondrial respiration, treatment with S100A8/A9 did not alter the muscle capillary content or muscle contractile force, assessed ex vivo in an isolated organ bath. A possible explanation for these observations might be related to the magnitude of mitochondrial impairment. Treatment with S100A8/A9 at 50 μg/kg body weight produced a modest (~10%) reduction in mitochondrial respiration at higher energy demands. The magnitude of difference in mitochondrial respiration between the placebo- and rS100A8/A9-treated mice at lower levels of energy demand (e.g., ΔG\text{ATP}, 63.8 kJ/mol) was much <10% (Fig 5). Therefore, future studies are warranted to explore the dose-dependent effects of S100A8/A9 in ischemic limb pathology.

The present study had several limitations that warrant discussion. First, the analyses involving CLTI patients should be considered preliminary because the sample size was limited and the patient phenotyping was not sufficiently detailed. Future cohort studies with larger patient numbers are required to examine the link between the serum and muscle levels of S100A8/A9 and CLTI disease severity. Additionally, the percentage of female participants in the present study was low; however, CLTI is prevalent in both male and female older adults. Second, although the proteomics analysis was considered robust, the limited specimen size for the non-PAD controls precluded our ability to validate protein levels using a different technique. However, we were able to confirm that the mRNA levels of S100A8/A9 were elevated, consistent with the proteomic results. Third, we did not perform analyses to determine the degree of systemic inflammation in the participants in the present study. Considering that S100A8/A9 levels are increased during states of inflammation, these analyses would be important to consider for both non-PAD and CLTI patients and could affect the interpretation of our preliminary clinical data. Fourth, the preclinical model we used was of C57BL6J mice, which are known to be resistant to limb ischemia and not adequately representative of CLTI. The C57BL6J strain was chosen because ischemia-sensitive strains, such as BALB/c, display severe
ischemic muscle mitochondrial pathology after induction of hindlimb ischemia, such that detecting exacerbation of the pathology with exogenous S100A8/A9 treatment could have proved difficult. Furthermore, mice used in the present study were male only, young in age, and free of comorbid diseases such as diabetes, hypertension, hyperlipidemia, obesity, and renal disease, all of which are likely to increase the likelihood of systemic inflammation. Thus, they do not model the CLTI patient characteristics well. Another consideration is that samples from the CLTI patients were obtained from the gastrocnemius muscle. However, in our mouse experiment, it was not possible to perform all the analyses using the gastrocnemius muscle because this entire muscle was needed for the mitochondrial isolation procedures, and other functional measures were not possible using this same muscle.

CONCLUSIONS
In the present study, we found that the S100A8 and S100A9 protein levels were elevated in the limb muscle of CLTI patients and that these levels were strongly associated with the degree of mitochondrial impairment. The treatment of mice with rS100A8/A9 impaired limb perfusion recovery and mitochondrial function after hindlimb ischemia. These findings highlight the possible role of S100A8/A9 in ischemic limb pathology and indicate that future studies examining the therapeutic potential of pharmacologic blockades of these inflammatory mediators are warranted.

AUTHOR CONTRIBUTIONS
Conception and design: ZS, TR
Analysis and interpretation: ZS, VM, NB, RK, NV, TT, KV, SB, SS, GV, TR
Data collection: ZS, VM, NB, RK, NV, TT, KV, SB, SS, GV, TR
Writing the article: ZS, TR
Critical revision of the article: ZS, VM, NB, RK, NV, TT, KV, SB, SS, GV, TR
Final approval of the article: ZS, VM, NB, RK, NV, TT, KV, SB, SS, GV, TR
Statistical analysis: ZS, TR
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