How to Cite this article: Brent Momb, Edwin Patino, Oleh Akchurin, and Mark Miller, Iron supplementation improves skeletal muscle contractile properties in mice with CKD, *Kidney360*, Publish Ahead of Print, 10.34067/KID.0004412021

**Article Type:** Original Investigation

**Iron supplementation improves skeletal muscle contractile properties in mice with CKD**

**DOI:** 10.34067/KID.0004412021

Brent Momb, Edwin Patino, Oleh Akchurin, and Mark Miller

**Key Points:**
*CKD in mice decreased single skeletal muscle fiber force production independent of fiber size.

*Iron supplementation in CKD mice improved aspects of contractile function, indicating CKD myopathy was partially mediated by iron imbalance.

*Our findings in mice suggest that timely correction of iron imbalance and anemia may improve muscle fiber function in patients with CKD.

**Abstract:**
Background: Chronic kidney disease (CKD) patients frequently have compromised physical performance, which increases their mortality; however, their skeletal muscle dysfunction has not been characterized at the single fiber and molecular levels. Notably, interventions to mitigate CKD myopathy are scarce. Methods: The impact of CKD in the absence and presence of iron supplementation on the contractile function of individual skeletal muscle fibers from the soleus and extensor digitorum longus muscles was evaluated in 16 week old mice. CKD was induced by adenine diet and iron supplementation was by weekly iron dextran injections. Results: Maximally activated and fatigued fiber force production was decreased 24-52% in untreated CKD, independent of size, by reducing strongly-bound myosin-actin cross-bridges and/or decreasing myofilament stiffness in myosin heavy chain (MHC) I, IIA and IIB fibers. Additionally, myosin-actin interactions in untreated CKD were slower for MHC I and IIA fibers and unchanged or faster in MHC IIB fibers. Iron supplementation improved anemia and did not change overall muscle mass in CKD mice. Iron supplementation ameliorated CKD-induced myopathy by increasing strongly-bound cross-bridges, leading to improved specific tension, and/or returning the rate of myosin-actin interactions towards or equivalent to control values in MHC IIA and IIB fibers. Conclusions: Skeletal muscle force production was significantly reduced in untreated CKD, independent of fiber size, indicating that compromised physical function in patients is not solely due to muscle mass loss. Iron supplementation improved multiple aspects of CKD-induced myopathy, suggesting that timely correction of iron imbalance may aid in ameliorating contractile deficits in CKD patients.

**Disclosures:** O. Akchurin reports the following: Other Interests/Relationships: ASN and ASPN member. The remaining authors have nothing to disclose.

**Funding:** HHS | NIH | National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK); K08 / DK114558; CU | Weill Cornell Medical College (Weill Cornell); Rohr Family Clinical Scholar Award

**Author Contributions:** Brent Momb: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Writing - original draft; Writing - review and editing Edwin Patino: Resources; Writing - review and editing Oleh Akchurin: Funding acquisition; Resources; Writing - review and editing Mark Miller: Conceptualization; Formal analysis; Investigation; Methodology; Resources; Supervision; Writing - original draft; Writing - review and editing

**Data Sharing Statement:** All data is included in the manuscript and/or supporting information.

**Clinical Trials Registration:**

**Registration Number:**

**Registration Date:**

Copyright 2022 by American Society of Nephrology.
The information on this cover page is based on the most recent submission data from the authors. It may vary from the final published article. Any fields remaining blank are not applicable for this manuscript.
Iron supplementation improves skeletal muscle contractile properties in mice with CKD

Brent A. Momb\textsuperscript{a}, Edwin Patino\textsuperscript{b}, Oleh M. Akchurin\textsuperscript{cd*}, Mark S. Miller\textsuperscript{a*}

\textsuperscript{a} Department of Kinesiology, University of Massachusetts, Amherst, MA, United States
\textsuperscript{b} Joan and Sanford I. Weill Department of Medicine, Division of Nephrology and Hypertension, Weill Cornell Medicine, New York, NY, USA
\textsuperscript{c} Department of Pediatrics, Division of Pediatric Nephrology, Weill Cornell Medicine, New York, NY, USA
\textsuperscript{d} New York-Presbyterian Hospital, New York, NY, USA

* These authors contributed equally

Corresponding Author:

Brent A. Momb
Department of Kinesiology
University of Massachusetts
30 Eastman Ln.
Amherst, MA, 01003, United States
Phone: (509)496-5799
Email: bmomb@umass.edu
Key Points

- CKD in mice decreased single skeletal muscle fiber force production independent of fiber size.
- Iron supplementation in CKD mice improved aspects of contractile function, indicating CKD myopathy was partially mediated by iron imbalance.
- Our findings in mice suggest that timely correction of iron imbalance and anemia may improve muscle fiber function in patients with CKD.
Abstract

**Background** Chronic kidney disease (CKD) patients frequently have compromised physical performance, which increases their mortality; however, their skeletal muscle dysfunction has not been characterized at the single fiber and molecular levels. Notably, interventions to mitigate CKD myopathy are scarce.

**Methods** The impact of CKD in the absence and presence of iron supplementation on the contractile function of individual skeletal muscle fibers from the soleus and extensor digitorum longus muscles was evaluated in 16 week old mice. CKD was induced by adenine diet and iron supplementation was by weekly iron dextran injections.

**Results** Maximally activated and fatigued fiber force production was decreased 24-52% in untreated CKD, independent of size, by reducing strongly-bound myosin-actin cross-bridges and/or decreasing myofilament stiffness in myosin heavy chain (MHC) I, IIA and IIB fibers. Additionally, myosin-actin interactions in untreated CKD were slower for MHC I and IIA fibers and unchanged or faster in MHC IIB fibers. Iron supplementation improved anemia and did not change overall muscle mass in CKD mice. Iron supplementation ameliorated CKD-induced myopathy by increasing strongly-bound cross-bridges, leading to improved specific tension, and/or returning the rate of myosin-actin interactions towards or equivalent to control values in MHC IIA and IIB fibers.

**Conclusions** Skeletal muscle force production was significantly reduced in untreated CKD, independent of fiber size, indicating that compromised physical function in patients is not solely due to muscle mass loss. Iron supplementation improved multiple aspects of CKD-induced myopathy, suggesting that timely correction of iron imbalance may aid in ameliorating contractile deficits in CKD patients.
Introduction

The decline in physical function from chronic kidney disease (CKD) is well predicted by glomerular filtration rate.\(^1\) Disease progression compromises the ability to perform activities of daily living and impaired physical function strongly predicts mortality in CKD patients.\(^2\) Frailty from muscle atrophy in CKD is multifactorial from malnutrition, decreased physical activity, cell senescence, and mitochondrial dysfunction.\(^3\) Importantly, reduced skeletal muscle mass does not completely explain poor physical function as whole muscle strength, defined as force production per body weight, is lower in CKD patients.\(^1\) These findings suggest that the underlying molecular contractile mechanisms, such as myofilament properties and myosin-actin interactions, are likely altered in CKD. In support of this hypothesis, single fiber force production per muscle size was reduced in an animal model of CKD; however, only one myosin heavy chain (MHC) isoform, MHC IIX, was examined in this study\(^4\) and the other functionally important isoforms, MHC I, IIA, and IIB, may respond differently to CKD. Understanding the molecular mechanisms behind the reduced skeletal muscle function in CKD is an important step in developing interventions to maintain physical function in patients.

Iron deficiency (absolute or, more commonly, functional) and anemia are common in CKD patients.\(^5\) CKD patients with anemia have greater fatigue\(^6\) and physical function impairment,\(^7\) while normalization of hemoglobin leads to improved function.\(^8\) Since sufficient iron and oxygen are essential in skeletal muscle for oxidative pathways, this may partially explain the additional decrements in physical function at the whole-body level in CKD patients with anemia.\(^5\) Contractile function of skeletal muscle may be altered through iron deficiency alone due to protein degradation through AMPK.\(^9\) Thus, one of our objectives was to determine if correction of functional iron deficiency and anemia in CKD would improve contractile function of skeletal muscle fibers.
Fiber contractile function is commonly examined under conditions that produce maximal activation and force production; however, other relevant \textit{in vivo} environments, such as fatigue and reduced calcium (Ca\textsuperscript{2+}) release, may be altered with CKD. Fatigue, or the decrease in whole skeletal muscle force or power production with repeated contractions, is exacerbated in CKD from greater accumulation of metabolic byproducts, specifically phosphate (P\textsubscript{i}) and hydrogen (H\textsuperscript{+}) ions.\textsuperscript{10} Elevated P\textsubscript{i} and H\textsuperscript{+} reduce the force and power generation of skeletal muscle fibers,\textsuperscript{11} in part from their large effect on the myosin-actin cross-bridge cycle.\textsuperscript{12} CKD may alter the sensitivity of skeletal muscle proteins to metabolic byproducts as reducing pH alone caused larger decreases in single fiber specific tension in an animal model with CKD.\textsuperscript{4} However, no prior studies have addressed muscle fatigue in CKD by altering both P\textsubscript{i} and H\textsuperscript{+} in single fibers. Ca\textsuperscript{2+} plays an important role in regulating skeletal muscle contractile function by binding to the troponin-tropomyosin complex and allowing myosin-actin cross-bridge formation and force generation. Ca\textsuperscript{2+} handling may be impaired in CKD as indicated by higher resting [Ca\textsuperscript{2+}] levels in skeletal muscle.\textsuperscript{13} As alterations in Ca\textsuperscript{2+} responsivity may cause declines in whole muscle performance,\textsuperscript{14} understanding if/how skeletal muscle Ca\textsuperscript{2+} sensitivity is modified by CKD is valuable.

This study was designed to examine the cellular and molecular contractile function of single fibers in an animal model of CKD and the effect of iron supplementation to counter the loss of skeletal muscle function. The effects of CKD and iron supplementation on single fiber properties were examined using three \textit{ex vivo} environments, maximal and sub-maximal Ca\textsuperscript{2+} activation as well as fatigue, as models for the physiologic conditions affecting muscle \textit{in vivo}. 
Materials and Methods

Mice

At 8 weeks of age, male C56BL/6J mice were randomly assigned to three groups: control, chronic kidney disease (CKD), and CKD with iron supplementation (CKDFE). CKD was induced with a 0.2% adenine diet for 8 weeks, as previously described. Mice were housed in temperature-controlled (~22°C) standard cages on a 12 h light-dark cycle with ad-libitum food and water intake. CKDFE mice in addition to adenine diet received intraperitoneal injections of iron dextran at 0.5 g/kg once a week. The soleus and extensor digitorum longus (EDL) muscles were collected at euthanasia at 16 weeks of age. Blood was collected at euthanasia from the inferior vena cava. Blood urea nitrogen (BUN) was measured on the Beckman Coulter (Brea, CA) AU 680 analyzer. Hemoglobin was measured on the IDEXX Procyte DX analyzer (Westbrook, ME). Animal experiments were approved by the Institutional Animal Care and Use Committee of Weill Cornell Medicine and complied with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Experimental Solutions for Single Fiber Experiments

Our commonly used experimental preparation allows the bathing solutions to enter the skeletal muscle fiber, meaning the ion concentrations throughout the fiber are tightly controlled. Dissecting solution was 20 mM N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid (BES), 5 mM ethylene glycol-bis(2-amino-ethylether)-N,N,N',N'-tetraacetic acid (EGTA), 5 mM MgATP, 1 mM free Mg\(^{2+}\), 1 mM dithiothreitol (DTT) and 0.25 mM Pi with pH 7.0 and at pCa 8.0 (pCa = -log\(_{10}([\text{Ca}^{2+}])\)). Skinning solution was 170 mM potassium propionate, 10 mM imidazole, 5 mM EGTA, 2.5 mM MgCl\(_2\), 2.5 mM Na\(_2\)H\(_2\)ATP and protease inhibitor (Roche) with pH 7.0. Storage solution was identical to skinning solution, but with 1 mM sodium azide. Relaxing solution was the same as dissecting solution, but with 5 mM Pi, 15 mM creatine phosphate and 300 µl/ml of
creatine phosphokinase. Pre-activating solution was the same as relaxing solution, except at an EGTA concentration of 0.5 mM. Activating solution was the same as relaxing solution, but at pCa 4.5. Relaxing and activating solutions were mixed to achieve different pCa concentrations. BDM solution was the same as relaxing with the addition of 40 mM 2,3-butanedione monoxime (BDM). Fatiguing solution was the same as activating solution except with reduced pH (6.2) and higher P_i (30 mM). Rigor solution was the same as activating but without creatine phosphate, creatine phosphokinase, and ATP. All solutions were adjusted to an ionic strength of 175 mEq using sodium methane sulfate.

Muscle Tissue Processing

Muscle tissue for mechanical measurements was placed into cold (4°C) dissecting solution immediately upon harvesting. Soleus and extensor digitorum longus (EDL) muscles were tied to glass rods and placed in skinning solution, a low Ca^{2+} solution that begins the removal of the muscle fibers’ external membrane, for 24 hr at 4°C and then stored in storage solution with glycerol (50% v/v) at -20°C. The addition of glycerol creates an osmotic pressure gradient to further permeabilize the muscle fibers’ external membrane and keeps the solution from freezing and damaging the fibers. Mechanical measurements and sinusoidal analysis occurred within 3 weeks of the dissection. Muscle tissues for apoptosis evaluation were fixed in 4% paraformaldehyde, paraffin-embedded, cut and processed for terminal deoxynucleotidyl transferase (TdT) deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL). Staining was performed by the histology core of the Laboratory of Comparative Pathology at Weill Cornell Medicine.

Preparation for Single Fiber Measurements

On the day of a muscle function experiment, a section of the stored whole muscle was excised to create a muscle fiber bundle and placed in dissecting solution with 1% v/v Triton X-100 for 30
min at 4°C to remove more of the sarcolemma and sarcoplasmic reticulum. After initial skinning, the bundle was transferred to dissection solution, cut into ~1 mm long sections and forceps used to isolate single fibers, which had aluminum T-clips placed on both ends. Fibers were demembranated a second time for 30 min at 4°C (dissection solution with 1% v/v Triton X-100) to ensure no remnants of the sarcolemma were intact. Fiber top and side diameters were measured in dissecting solution to estimate the height-to-width ratio and subsequently transferred to the experimental apparatus containing relaxing solution at 15°C. The fiber was mounted in relaxing solution to the experimental apparatus by sliding the holes in the T-clips onto hooks attached to a piezo actuator linear motor (P-841.10, Physik Instrumente, Auburn, MA) and an Akers strain gauge (AE-801, SensorOne, Sausalito, CA) (Figure 1A). Sarcomere length was set to 2.65 µm and cross-sectional area (CSA) was determined by measuring fiber width on the apparatus’ compound microscope, estimating the height based upon the height-to-width ratio measured via microscope prior to mounting, and presuming fiber CSA is elliptical. The fiber was slackened completely, the force gauge zeroed, the fiber pulled back to its original length, allowed to equilibrate for 1 min, relaxed specific tension measured and then transferred to pre-activating solution for 30 s. At this point, fibers were either (1) placed into activating (pCa 4.5) or fatiguing solution, with specific tension measured at its plateau and sinusoidal analysis performed, or (2) placed into activating (pCa 4.5) solution and specific tension-pCa relationships, or (3) dynamic stiffness experiments performed in activating and rigor solutions, and then (4) placed into BDM solution and passive stretching experiments performed.

**Experimental Apparatus**

A custom built single fiber skeletal muscle mechanics apparatus was used, as previously described.16 Briefly, an aluminum bath plate of 13 wells (~100 µl each) was made to hold experimental solutions and a single large chamber (~450 µl) for mounting the fiber onto the force and linear motor hooks. Once a fiber was mounted in solution, the bath plate was moved
vertically, to move the fiber out of a chamber, and slid horizontally within a trough, underneath the fixed motor and force gauge, allowing the fiber to be moved (~1 s) between chambers and different solutions. The bathing solutions were maintained at a constant temperature by circulating cooling solution through channels milled into the chamber walls. The bathing solution assembly was mounted to an inverted microscope (Zeiss Invertoscope) with a video camera (BFLY-U3-23S6m-C, Point Grey Research Inc., Richmond, British Columbia, CA) and custom video analysis software (ImageJ) that enabled precise measurements of fiber dimensions and sarcomere length.

**Single Fiber Mechanical Measurements**

Myosin-actin cross-bridge mechanics and kinetics as well as properties of the myofilaments were derived using sinusoidal analysis, as previously described, over frequencies ranging from 1-200 Hz at 25°C. Briefly, small-amplitude, sinusoidal strain (normalized length, or length per change in length) was applied to one end of the fiber, while the sinusoidal stress output (normalized force, or force per cross-sectional area) was measured at the other end of the fiber (Figure 1A). The stress output ($\sigma$) can be divided into its elastic ($\sigma_e$) and viscous ($\sigma_v$) components by determining the magnitudes of the in-phase and out-of-phase components (0° and 90° with respect to strain), and the elastic ($E_m$) and viscous ($V_m$) modulus calculated by dividing maximal elastic (amplitude of $\sigma_e$) or viscous (amplitude of $\sigma_v$) stress by strain ($\varepsilon_{amp}$, 0.125% of muscle length unless otherwise noted) (Figure 1B). The elastic and viscous moduli are the real and imaginary parts of the complex modulus, the ratio of the stress output to the strain. Sinusoidal analysis results can be related to specific steps in the cross-bridge cycle by fitting the complex modulus with the following six parameter equation:

$$Y(\omega) = A(i\omega/\alpha)^k - B\omega/(2\pi b + i\omega) + C\omega/(2\pi c + i\omega)$$
where $\omega = 2\pi f$ in $s^{-1}$, $A$, $B$ and $C$ are magnitudes expressed in N/mm$^2$, $2\pi b$ and $2\pi c$ are characteristic rates expressed in $s^{-1}$, $i = -1^{1/2}$, $\alpha = 1 \text{ s}^{-1}$ and $k$ is a unitless exponent. This analysis yields three characteristic processes, $A$, $B$ and $C$, which relate to various mechanical ($A$, $B$, $C$ and $k$) and kinetic ($2\pi b$ and $2\pi c$) properties of the cross-bridge cycle (Figure 1C), as previously described. Nyquist plots, or viscous versus elastic modulus, show the stress response of the fiber to strain over a range of frequencies, which can be fit using the six-parameter equation above (Figure 1D). The $A$-process ($A$ and $k$) has no enzymatic dependence and, under Ca$^{2+}$-activated conditions, reflects the viscoelastic mechanical response of the structural elements of muscle fiber (the myofilament lattice stiffness and attached myosin heads in series). The parameter $A$ indicates the magnitude of the viscoelastic modulus, whereas $k$ represents the degree to which these viscoelastic magnitudes are purely elastic ($k = 0$) or purely viscous ($k = 1$). As the physical meaning behind $A$ and $k$ can be difficult to understand, the parameter $A$ was instead separated into its real ($A$-elastic) and imaginary ($A$-viscous) components using the following equation:

$$A(i\omega/\alpha)^k = A\text{-elastic}(\omega/\alpha)^k + iA\text{-viscous}(\omega/\alpha)^k$$

where $A\text{-elastic} = A \cos(k \pi/2)$ and $A\text{-viscous} = A \sin(k \pi/2)$. The $B$- and $C$-process magnitudes ($B$ and $C$) are proportional to the number of strongly bound myosin-actin interactions and/or the stiffness of the cross-bridges. As the $B$ and $C$ process magnitudes produce qualitatively near-identical results, only the $B$ values are shown. The frequency portion of the $B$-process ($2\pi b$) is interpreted as the rate of myosin transition from the weakly to strongly bound state, or the (apparent) rate of myosin force production. The frequency portion of the $C$-process ($2\pi c$) represents the cross-bridge detachment rate, or, in other words, the inverse $(2\pi c)^{-1}$ is the mean myosin attachment time to actin, $t_{on}$. 
Specific Tension-pCa Relationships

After initial activation to test for fiber integrity, fibers were placed in relaxing solution, sarcomere length set to 2.65 µm, slackened to set zero specific tension, stretched back to original length (sarcomere length = 2.65 µm) and allowed to equilibrate for 1 min. Relaxed specific tension was measured and the fiber transferred to pre-activating solution for 30 s and moved to solutions with progressively greater Ca$^{2+}$ levels (pCa 7.0, 6.5, 6.25, 6.1, 6.0, 5.9, 5.75, 5.5, 5.0, 4.5) with specific tension measured at each plateau. Normalized specific tension was calculated for individual fibers by the following equation:

$$(x - \text{specific tension at pCa 7.0}) / (\text{specific tension at pCa 4.5} - \text{specific tension at pCa 7.0})$$

where $x$ is the tension at a given Ca$^{2+}$ level.

Normalized force recordings were fit to the Hill equation:

$$[\text{Ca}^{2+}]^h / ([\text{Ca}^{2+}]_{50}^h + [\text{Ca}^{2+}]^h)$$

where $[\text{Ca}^{2+}]_{50} = \text{calcium concentration at half activation}$, pCa$_{50} = -\log [\text{Ca}^{2+}]_{50}$, and $h = \text{Hill coefficient}$.

Dynamic Stiffness Measurements

A subset of fibers underwent maximal Ca$^{2+}$ and rigor activation. At the tension plateau for each activation condition, sinusoidal length perturbations (50 cycles of 0.125% fiber length at 360 Hz) were imposed to provide dynamic stiffness measurements. The amplitude of dynamic stiffness at maximal Ca$^{2+}$-activation is proportional to the number of heads bound under maximal activation, while the amplitude of dynamic stiffness in rigor is proportional to the total number of myosin heads available to bind to actin, or the total cross-bridge number, with the assumption that all myosin heads bind to actin in rigor. Thus, the ratio of maximal Ca$^{2+}$-activated to rigor stiffness provides an estimate of the fraction of myosin heads available that bind to actin.
Passive Stretching Dynamic Stiffness Measurements

A subset of fibers underwent stretching experiments under relaxed conditions with BDM, which places the myosin heads in a pre-power stroke, non-force producing state to remove their contribution to specific tension and dynamic stiffness. Sarcomere length was set to 2.65 µm and a single sinusoidal length perturbation (50 cycles of 0.25% fiber length at 100 Hz) was applied to determine dynamic stiffness. The fiber was then stretched to 2.8, 3.0, 3.2, 3.4, 3.6, 3.8 and 4.0 µm with sinusoidal length perturbations at each interval after a tension plateau was reached.

Myosin Heavy Chain Isoform Composition

Following sinusoidal analysis measurements, single fibers were placed in 30 µl loading buffer, heated for 2 min at 65°C and stored at -80°C until determination of myosin heavy chain (MHC) isoform composition by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to identify fiber type, as described, with minor modifications to achieve better separation between the four MHC isoforms expressed in mouse skeletal muscle. Specifically, the resolving gel was comprised of 8% acrylamide/bis-30% glycerol (w/v) and gels were run at 70 V for 1 h followed by 275 V for 26 h.

Statistical Analysis

Data are expressed as mean ± SE and the statistical tests used for each experiment are provided in Supplemental Table 1. As there are multiple observations of variables within the same mouse, a linear mixed model was used, including a random effect to account for clustering of observations within mice. *P*-values ≤ 0.05 were considered statistically significant. Statistical analyses were conducted using SPSS for Windows version 27.0 (IBM, Armonk, NY).
Results

Chronic Kidney Disease Characteristics

CKD and CKDFE mice had similar reductions in body, muscle, and kidney mass compared to controls (Table 1). Iron supplementation improved anemia in CKDFE mice compared to untreated CKD (Table 1). Body weight was similar at 8 weeks of age (at the beginning of the experimental period) for all groups, but decreased 29-33% over the duration of the adenine diet for CKDFE and CKD while body mass was non-significantly increased (10%) over this time period in control mice (Supplemental Figure 1).

Maximal Ca\(^{2+}\)-activated Properties

The effects of CKD and CKDFE on the cellular (force, CSA and specific tension) and molecular (myofilament properties and myosin-actin cross-bridge kinetics) contractile properties of single fibers were examined by MHC isoform as MHC is the primary determinant of fiber functional characteristics. All MHC I and IIA fibers were from the soleus and all MHC IIB fibers were from the EDL.

Force, CSA and Specific Tension. Average specific tension (force per CSA) was reduced by 36-51% for CKD and CKDFE for MHC I, IIA and IIB fibers, although CKDFE fibers had increased specific tension compared to CKD for MHC IIA and IIB fibers (Figure 2A). These results indicate iron supplementation improved force production in fast-contracting fiber types (MHC IIA and IIB), but not slow-contracting (MHC I). Specific tension findings were consistent across fiber types, but changes in force and CSA were fiber type dependent (Figure 2B-C). MHC I fibers in CKD and CKDFE had similar force production to controls, but were hypertrophied. MHC IIA CKD and CKDFE fibers had reduced force production, but hypertrophy that occurred with CKD was not present in CKDFE. MHC IIB fibers in CKD and CKDFE were atrophied, but force production was reduced less in CKDFE than CKD. For each fiber type, the relationship between
single fiber force production and size was further examined by plotting the force versus CSA for individual fibers and performing a linear regression for each group (Figure 2D-F). In general, CKD and CKDFE force-CSA relationships showed lower slopes and intercepts for MHC I, IIA and IIB fibers. The only exceptions were in MHC I fibers where CKD had a similar slope and CKDFE had a higher intercept compared to control. These plots indicate that for a given size, force production is reduced for CKD and CKDFE.

Myofilament Properties and Myosin-Actin Cross-Bridge Kinetics. Representative sinusoidal analysis data and six parameter equation curve fits, which are used to determine myofilament properties and myosin-actin cross-bridge kinetics, are shown in Figure 1D. The number of strongly bound cross-bridges and/or the cross-bridge stiffness ($B$ parameter) was lower in CKD and CKDFE for MHC I, IIA and IIB fibers, although CKDFE fibers had increased values compared to CKD for MHC IIA and IIB fibers (Figure 3A). We interpret the large reduction in specific tension and $B$ to be from a reduction in the number of strongly-bound cross-bridges as recent modeling results indicate that cross-bridge stiffness only modestly alters isometric force production. These results indicate that iron supplementation increased strongly bound cross-bridges in fast-contracting fiber types compared to CKD. The $A$-elastic and $A$-viscous parameters reflect the elastic and viscous properties of the myofilament lattice structure and the attached myosin heads in series. $A$-elastic and $A$-viscous were unchanged in MHC I fibers, but decreased in CKD and CKDFE MHC IIA and IIB fibers, with an improvement in $A$-viscous with iron supplementation compared to CKD in MHC IIB fibers (Figure 3B-C). The myosin rate of force production, or $2\pi b$, in CKD and CKDFE was slowed in MHC I and IIA fibers, but unchanged in MHC IIB fibers (Figure 3D). Myosin attachment time, or $t_{on}$, in CKD and CKDFE was unchanged in MHC I and MHC IIB, but longer in MHC IIA (Figure 3E). Overall, if cross-bridge kinetics ($2\pi b$ or $t_{on}$) changed with CKD and CKDFE, they were slower in MHC I and IIA and unchanged in MHC IIB fibers. Notably, CKDFE MHC IIA fibers had faster cross-bridge
kinetics than CKD showing iron supplementation partially ameliorated the effects of CKD. As such, contractile velocities of single fibers could be increased in response to iron supplementation, as $t_{on}$ is inversely proportional to velocity.\textsuperscript{19}

\textit{Relationship to Hemoglobin Levels}

Iron supplementation increased hemoglobin as well as specific tension and the number of myosin heads strongly-bound to actin in MHC IIA and IIB fibers and cross-bridge kinetics in MHC IIA fibers under maximal Ca\textsuperscript{2+}-activating conditions compared to untreated CKD mice. To better understand the relationship between hemoglobin and contractile function, we examined the association between cellular/molecular parameters and hemoglobin. Increased specific tension and $B$ in MHC IIA and IIB fibers and faster cross-bridge kinetics in MHC IIA fibers were correlated with hemoglobin (Figure 4A-F). These findings indicate improvements in hemoglobin are associated with increased force production, likely due to an increased number of strongly-bound myosin and potentially faster contractile velocities, as a consequence of faster cross-bridge kinetics.\textsuperscript{19,20}

\textit{Passive and Rigor Properties}

Specific tension and dynamic stiffness were examined at various sarcomere lengths in relaxing solution containing BDM, placing the myosin heads are in a pre-power stroke, non-force producing state,\textsuperscript{21} producing measures of the myofilament lattice properties. As expected, specific tension and stiffness rose with increasing sarcomere length in all three fiber types (Figure 5). Specific tension and dynamic stiffness in CKD fibers remained similar to control in MHC I fibers, but decreased 32-60\% in MHC IIA and 42-68\% in IIB fibers, showing that CKD greatly reduced the myofilament lattice stiffness in fast-contracting fibers. Hence, the large decreases in $A$-elastic and $A$-viscous with CKD are due, in a large part, to reduced myofilament lattice stiffness.
The ratio of maximal $\text{Ca}^{2+}$-activated stiffness to rigor stiffness was increased with CKD in MHC I and IIA fibers (Figure 6A), indicating a greater percentage of the total number of myosin heads available were strongly bound under maximal activation. However, rigor and maximum $\text{Ca}^{2+}$-activated stiffnesses were reduced with CKD in MHC I, IIA and IIB fibers (Figure 6B-C). In MHC I fibers that had similar myofilament stiffness between groups (Figure 5D), these reductions indicate the total number of available myosin heads that can bind actin were reduced, leading to reduced strongly-bound heads under maximum $\text{Ca}^{2+}$-activation. In MHC IIA and IIB fibers, the decreased values may be explained by the reduced myofilament stiffness between CKD and control (Figure 5E-F), or a reduction in available myosin heads.

**Fatigued Properties**

*In vivo*, elevated intracellular $\text{P}_i$ and $\text{H}^+$ are the hallmarks of muscle fatigue. In order to understand how skeletal muscles in CKD are affected by fatigue, and to elucidate the effect of iron supplementation on muscle fatigue, we simulated this characteristic fatigue environment *in vitro* by reducing muscle pH (7.0 to 6.2) and increasing $\text{P}_i$ (5 mM to 30 mM) in the experimental solutions. Specific tension decreased from maximal-activated (Figure 3A) to fatigued (Figure 7A) for each condition (control, CKD and CKDFE) and fiber type (MHC I, IIA and IIB) due to reduced force as CSA remained unchanged, indicating force was reduced independent of fiber size (Supplemental Figure 2). Like maximal $\text{Ca}^{2+}$-activated (Figure 2A), fatigued specific tension was reduced in both CKD and CKDFE groups for MHC I, IIA and IIB fibers (Figure 7A). The relative drop in specific tension with fatigue was less for CKD (24%) than controls (42%) in MHC I fibers ($p = 0.024$), but similar in MHC IIA (38-41%) and IIB (50-52%) fibers. Molecular parameters dictating contractile function in controls and CKD showed similar patterns during maximal $\text{Ca}^{2+}$-activation (Figure 3A-E) and fatigue (Figure 7B-F). The exceptions were that during fatigue, $A$-elastic and $A$-viscous were decreased as well as $t_{on}$ increased in CKD in MHC I fibers, $B$ was unchanged in CKD and controls in MHC IIA fibers, and cross-bridge kinetics
were faster in MHC IIB fibers. Iron supplementation returned $B$ in MHC IIB fibers and cross-bridge kinetics in MHC IIA and IIB fibers back to control values, indicating that iron supplementation was able to improve molecular interactions during fatigue.

**Submaximal $Ca^{2+}$-activated Properties**

The $Ca^{2+}$ response of muscle in controls, CKD and CKDFE was examined to understand the effects of altered *in vivo* $Ca^{2+}$ handling, which can occur under variety of conditions, including Vitamin D deficiency, aging, and potentially CKD. Specific tension across all calcium concentrations (pCa 7-4.5) was lower in CKD and CKDFE for MHC I, IIA and IIB fibers (Figure 7A-C). Submaximal specific tension (pCa 6.1-5.75) was ordered control > CKD > CKDFE in MHC I, IIA and IIB fibers, except for pCa 6.1 in MHC IIB fibers where CKD = CKDFE (Figure 8A-C). Submaximal normalized specific tension was ordered controls = CKD > CKDFE in MHC I fibers and controls > CKD > CKDFE in MHC IIA and IIB fibers (Figure 8D-F). Based upon the normalized specific tension curves, $Ca^{2+}$ sensitivity (pCa$_{50}$) was ordered control = CKD > CKDFE in MHC I and IIA fibers and controls > CKD > CKDFE in IIB fibers, showing that sensitivity was reduced the most by iron supplementation (Table 2). The Hill coefficient (h), a measure of the steepness of the force response to $[Ca^{2+}]$, was decreased in CKDFE compared to control and/or CKD for MHC I and IIA fibers, showing that cooperativity was lower for CKDFE.

**TUNEL Labeling**

To identify if apoptosis could be a mechanism behind the decreased muscle mass in CKD and CKDFE (Table 1), we performed TUNEL labeling of skeletal muscle sections, which was negative (Supplemental Figure 3). Thus, apoptosis of myocytes was likely not a major contributor to muscle loss in this model of CKD.
Discussion

This study is the first to comprehensively assess cellular and molecular mechanisms of CKD-induced contractile myopathy. Maximally activated and fatigued fibers from mice with CKD had reduced force generation due to reduced strongly-bound cross-bridges and/or decreased myofilament stiffness in MHC I, IIA and IIB fibers. Myosin-actin interactions (cross-bridge kinetics) with CKD were slower for MHC I and IIA fibers, but unchanged or faster in MHC IIB fibers under maximal activation and fatiguing conditions. Ca$^{2+}$ sensitivity was only slightly reduced in MHC IIB fibers in CKD mice. Iron supplementation ameliorated myopathy associated with CKD, indicating that disrupted iron homeostasis and anemia in CKD likely contribute to muscle dysfunction.

Since molecular level interactions within individual muscle fibers dictate cellular to whole muscle to whole body performance, our results provide novel insights into the underlying mechanisms of compromised physical function in CKD. Previous studies have reported reduced whole muscle isometric torque production in CKD patients and an animal model. Our work builds upon these findings by identifying molecular mechanisms behind reduced force production in single skeletal muscle fibers. Frailty is prevalent in CKD patients and can present as slowed gait speed, which is a determinant of poor physical function and mortality. Slower cross-bridge kinetics are responsible for decreased single fiber contractile velocity and thus may be implicated in slower gait speeds in patients. Slower cross-bridge kinetics could also lead to the longer muscle relaxation rates described in dialysis patients. The differential findings from various MHC isoforms may be due to muscle-specific loading or function as MHC I and IIA fibers were present in soleus and MHC IIB fibers in EDL in mice. In humans, the soleus and EDL muscles are utilized for plantar flexion and dorsiflexion, which
are critically important for ambulation.\textsuperscript{34} Notably, in our experiments CKD produced striking alterations in the performance of MHC I and IIA fibers, which are most commonly expressed in humans. Overall, our results suggest that decreased myofilament stiffness, reduced myosin-actin cross-bridges, and slowed cross-bridge kinetics should be examined in patients with CKD as they likely have a major role in the loss of physical function.

Iron supplementation alleviated anemia in CKD mice. As non-renal anemia is associated with muscle weakness in older adults,\textsuperscript{35} anemia in CKD may exacerbate the disease-associated myopathy, however direct evidence is lacking. Anemia is common in CKD patients and results in greater fatigue\textsuperscript{6} and physical impairment,\textsuperscript{7} possibly due to decreases in skeletal muscle performance. In our study, CKD-induced anemia was partially reversed using iron supplementation and reduced contractile deficits in MHC IIA and IIB fibers, suggesting that iron may benefit fast-contracting fibers in CKD patients. This is particularly important because fast-contracting fibers produce higher forces\textsuperscript{26} and velocities\textsuperscript{17} than slower-contracting fibers and support rapid movements while relying on anaerobic glycolysis. Improvements in fast-contracting MHC IIA and IIB fibers by iron supplementation may be explained by their lower baseline myoglobin\textsuperscript{36} and overall oxygenation\textsuperscript{37} compared to MHC I fibers. Myoglobin is an iron containing antioxidant that scavenges metabolic byproducts\textsuperscript{38} and fast-contracting fibers may have lower baseline levels, making them more susceptible to iron deficiency during CKD. Higher tissue oxygenation through increased hemoglobin and/or myoglobin enhances skeletal muscle protein synthesis\textsuperscript{39} and nitric oxide production,\textsuperscript{40} potentially increasing contractile velocity.\textsuperscript{41} Although iron supplementation generally improved contractile performance, Ca\textsuperscript{2+} sensitivity was reduced. However, this may not be relevant to patients with CKD as resting skeletal muscle [Ca\textsuperscript{2+}] is typically elevated in CKD.\textsuperscript{13} Improvements in contractile function from iron
supplementation could also be due to enhanced physical activity due to mitigation of the anemia-related physical impairments. As physical activity can improve physical function and reduce fatigue, future work should incorporate assessment of physical activity in studies of muscle function in CKD.

The magnitude of effects that iron supplementation exerted on many parameters of single fiber performance are comparable or exceeding those reported for effective interventions targeting muscle function in patients with CKD. Indeed, CKD patients undergoing progressive resistance training were able to achieve an increase in knee extensor strength of 13-25%. These improvements in strength were associated with improved quality of life and physical function. Thus, these studies indicate improvements of 13-25% in knee extensor strength improve physical function in CKD patients. Assuming that an increase in single fiber force production per muscle size (specific tension) would translate to a similar improvement in force production at the whole muscle level, our findings that iron supplementation improves specific tension by 21-26% in MHC IIA and IIB fibers (Figure 2A) compared to CKD suggests that iron supplementation could provide a clinically relevant improvement in CKD patients. Another recent clinical study found that improvements in whole muscle power output of 9-10% were clinically meaningful in mobility-limited older adults. Power output, which is the product of force and velocity, is a key predictor of the age-related decrease in functional capacity. MHC IIA fibers showed a 18% decrease in myosin attachment time ($t_{on}$) with iron supplementation compared to CKD, which should increase single fiber contractile velocity and power output. Overall, our results fall into the range of potentially being clinically meaningful in terms of improving whole muscle functional performance.
Muscle force production was previously shown to be reduced in response to low pH, which is a key metabolic byproduct of fatigue, in an animal model of CKD in MHC IIX fibers. However, when simulating fatigue by decreasing pH and increasing P\textsubscript{i} in the bathing solutions surrounding and within the fiber, our findings show that the relative decrease in force with fatigue was less for CKD than control for MHC I and was similar for both groups in MHC IIA and IIB fibers. These results indicate that the force producing mechanisms in these fiber types may become resistant to the metabolic byproducts of P\textsubscript{i} and pH, possibly indicating a protective response of muscle fibers to CKD. Additionally, the different patterns in the response of the cross-bridge kinetics to fatigue with CKD that we observed, most notable in MHC I and IIB fibers, may account for alterations in contractile velocity.

Examining our results in the context of the sarcomeric determinants of force generation provides insights into the potential mechanisms responsible for the loss of specific tension in CKD. Under Ca\textsuperscript{2+} activated conditions, single fiber isometric force production is due to the number of strongly-bound myosin-actin cross-bridges, the force generated per cross-bridge and the stiffness of the sarcomeric elements that transmit the force. Lower force transmission, due to decreased stiffness of the myofilaments (i.e., thin (primarily actin), thick (primarily myosin) and titin) as well as other sarcomeric structures that anchor the filaments (i.e., Z-bands and M-lines) and link myofibrils (i.e., costameres), reduces the active force generated by the sarcomere. Under passive conditions, single fiber isometric force production is due to stiffness of the elements providing force transmission, except for the thin filament, as the strongly-bound cross-bridges have been removed. In the present study, active specific tension with CKD was reduced in all fiber types examined, while passive specific tension was reduced only in MHC IIA and IIB fibers (Figure 5A-C). These results indicate that sarcomeric protein alterations were fiber type specific, possibly due to their commonly expressing different isoforms of the myofilament, Z-
band and M-line proteins. Titin is a primary determinant of passive force and alters active properties, making this giant protein a strong candidate for the observed changes in MHC IIA and IIB fibers. Titin alters its stiffness through various means, including Ca$^{2+}$ binding, phosphorylation and oxidation, and can alter myofilament lattice spacing which affects myosin’s ability to bind actin. The loss of myofilaments and Z-band streaming observed in hemodialysis patients should decrease the number of strongly-bound cross-bridges and/or force transmission. Shorter thick filament length due to remodeling could also reduce active force generation as fewer myosin heads would be available to strongly bind to actin. Myosin and/or actin loss, titin modifications and/or thick filament shortening have been observed in disuse; however, direct data on these potential mechanisms in pre-hemodialysis CKD patients are lacking. CKD may additionally cause post-translational modifications of sarcomeric proteins, leading to altered properties. Thus, there are several plausible putative mechanisms which likely occur in combination, ultimately leading to the skeletal muscle force production loss in CKD. Our findings highlight an unmet need for future studies to examine these mechanisms, which will aid in development of CKD-specific therapeutic approaches.

Fiber CSA in CKD increased (17-73%) in MHC I and IIA fibers from the soleus and decreased (21%) in MHC IIB fibers from the EDL, despite significant weight loss (44-54%) in both muscles. Therefore, MHC IIB fiber atrophy may explain loss of EDL weight. However, the mechanism of soleus weight loss appears different because individual MHC I and IIA fiber size in CKD mice was either similar or larger than control mice (Figure 2C). These results indicate that fiber loss and hypertrophy of the remaining fibers are occurring with CKD in the soleus. This differential muscle response to CKD may be explained by the higher activation of the soleus during resting, standing, and walking than the EDL. Another proposed mechanism of fiber enlargement in CKD is edema; however, mice treated with adenine do not develop generalized edema.

Our
study highlights that fiber loss, which has not been directly examined in CKD animal models or patients to our knowledge, may have important translational and potentially therapeutic implications. Fiber size has received more attention in the literature, but no clear pattern has been established in patients with CKD, in part because studies commonly utilize reference values instead of controls. Even in the very few studies employing control groups, results include larger, unchanged, or smaller MHC I and II fibers from patients, although the largest study performed to date (n = 60 vs. 10-12 patients) reported larger fibers (26-33%), consistent with our findings. One important confounder that may explain the heterogeneity in fiber size in CKD is physical activity, which is highly variable in patients with CKD and should be assessed in future studies.

In conclusion, in this CKD model, CKD-induced myopathy was encompassed by the loss of myofilament stiffness, reduced myosin-actin cross-bridges, and slowed cross-bridge kinetics. These uremia-driven alterations are likely compounded by iron imbalance and anemia. Correction of functional iron deficiency and anemia improved skeletal muscle function in our model of CKD, which has direct translational potential for improving physical function in patients with CKD. Translation of these findings to human studies is warranted with testing of additional interventions, such as resistance exercise, to increase force production and restore cross-bridge kinetics of muscle fibers to improve physical function in patients with CKD.
Disclosures

O. Akchurin reports the following: Other Interests/Relationships: ASN and ASPN member. The remaining authors have nothing to disclose.

Funding

This work was supported in part by the National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases K08 DK114558 (OA) and Weill Cornell Medicine Rohr Family Clinical Scholar Award (OA).

Acknowledgements

The authors thank Chantalle Campbell, BA for help with data management. The authors appreciate the help of Maria Jiao, BSMT, histology technician from the Laboratory of Comparative Pathology for help with TUNEL staining.

Author Contributions

Brent Momb: Conceptualization(designed fiber experiments); Data curation; Formal analysis; Investigation; Methodology; Writing - original draft; Writing - review and editing. Edwin Patino: Resources(developed, maintained, and provided tissues and data for the mouse model); Writing - review and editing. Oleh Akchurin: Funding acquisition; Resources(developed, maintained, and provided tissues and data for the mouse model); Writing - review and editing. Mark Miller: Conceptualization(designed fiber experiments); Formal analysis; Investigation; Methodology;
Resources; Supervision; Writing - original draft; Writing - review and editing. All authors contributed to data interpretation and manuscript revision.

Data Sharing Statement
All data is included in the manuscript and/or supporting information.

Supplementary Materials
Supplemental Table 1. Statistical methods used for each experimental condition
Supplemental Figure 1. Body weight during the experimental period
Supplemental Figure 2. Single skeletal muscle fiber force and cross-sectional area (CSA) for control, CKD and CKDFE groups by fiber type and representative gels
Supplemental Figure 3. Assessment of apoptosis in skeletal muscle in three groups of mice
References


vertebrate striated muscle in antioxidant defense. *Comparative Biochemistry and Physiology: Biochemistry and Molecular Biology* 234: 9–17, 2019


Table 1. Disease characteristics in three groups of mice

<table>
<thead>
<tr>
<th></th>
<th>Control (n=10)</th>
<th>CKD (n=12)</th>
<th>CKDFE (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>28.4 ± 0.5</td>
<td>17.1 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17.3 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Soleus weight (mg)</td>
<td>22.8 ± 1.3</td>
<td>10.6 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.4 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>EDL weight (mg)</td>
<td>20.2 ± 1.4</td>
<td>11.4 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.1 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Left kidney weight (mg)</td>
<td>188.2 ± 8.2</td>
<td>126.1 ± 5.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>127.5 ± 10.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Right kidney weight (mg)</td>
<td>185.4 ± 4.7</td>
<td>111.1 ± 6.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>112.0 ± 3.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Body weight:Kidney weight ratio</td>
<td>6.5 ± 0.1</td>
<td>6.9 ± 0.2</td>
<td>7.0 ± 0.6</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>32.2 ± 1.7</td>
<td>77.5 ± 5.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>73.6 ± 2.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>13.9 ± 0.2</td>
<td>9.4 ± 0.2&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>11.3 ± 0.3&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SE.  
<sup>a</sup>significant difference (<i>p</i> < 0.05) compared to control,  
<sup>b</sup>significant difference compared to CKD,  
<sup>c</sup>significant difference compared to CKDFE.  
0.2% Adenine diet was started in CKD and CKDFE at 8 wk age, body weight was measured, blood collected, kidneys and muscles harvested at euthanasia at 16 wk age. CKD, chronic kidney disease; CKDFE; CKD plus weekly iron dextran administration. BUN, blood urea nitrogen.
Table 2. Ca\(^{2+}\) sensitivity (pCa\(_{50}\)), and force-pCa slope (Hill coefficients \(h\)) of myosin heavy chain (MHC) I, IIA, and IIB of single muscle fibers from control, CKD and CKDFE mouse groups.

<table>
<thead>
<tr>
<th>MHC</th>
<th>n</th>
<th>Control</th>
<th>CKD</th>
<th>CKDFE</th>
<th>Control</th>
<th>CKD</th>
<th>CKDFE</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>5/20/8</td>
<td>5.95 ± 0.04</td>
<td>5.97 ± 0.01</td>
<td>5.82 ± 0.04(^{a,b})</td>
<td>7.15 ± 1.66</td>
<td>5.30 ± 0.32</td>
<td>3.96 ± 0.35(^{a})</td>
</tr>
<tr>
<td>IIA</td>
<td>45/37/22</td>
<td>5.97 ± 0.01</td>
<td>5.92 ± 0.01</td>
<td>5.72 ± 0.02(^{a,b})</td>
<td>5.65 ± 0.39</td>
<td>5.35 ± 0.35</td>
<td>3.75 ± 0.36(^{a,b})</td>
</tr>
<tr>
<td>IIB</td>
<td>40/56/34</td>
<td>5.91 ± 0.01</td>
<td>5.85 ± 0.01(^{a,c})</td>
<td>5.73 ± 0.02(^{a,b})</td>
<td>4.51 ± 0.25</td>
<td>4.51 ± 0.18</td>
<td>4.39 ± 0.12</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SE. Values correspond to the normalized specific tension-pCa curves. \(n\) indicates number of fibers assessed. \(^{a}\) significant difference \((p < 0.05)\) compared to control, \(^{b}\) compared to CKD, \(^{c}\) compared to CKDFE. MHC, myosin heavy chain. C, control; CKD, chronic kidney disease; CKDFE, CKD plus weekly iron dextran administration.
Figure Legends

Figure 1. Single muscle fiber mechanical experiments, analysis and representative data for control and chronic kidney disease (CKD) mouse groups. (A) Sinusoidal strain input applied to one end of a Ca\(^{2+}\)-activated fiber produced a sinusoidal stress output measured at the other end. (B) Left: Fiber stress output, from 15 Hz strain input (strain amplitude or \(\varepsilon_{\text{amp}} = 0.125\%\)), divided into elastic (\(\sigma_e\)) and viscous (\(\sigma_v\)) components. Right: Nyquist plot, where each open circle represents the elastic and viscous moduli at each oscillation frequency (from 1 to 200 Hz), produced by sinusoidal analysis. Elastic and viscous moduli are calculated by dividing the maximal elastic (amplitude of \(\sigma_e\)) or viscous (amplitude of \(\sigma_v\)) stress by strain (\(\varepsilon_{\text{amp}}\)), with an example shown at 15 Hz. (C) Left: The six model parameters, used to fit the Nyquist plot, can be related to myosin-actin cross-bridge function and myofilament structural properties, and are paired into three different processes: the linear A-process (continuous line, described by parameters \(A\) and \(k\)), the semicircular B-process (dashed line, described by parameters \(B\) and \(b\)) and the C-process (dash-dot line, described by parameters \(C\) and \(c\)). Right: Six parameter model equation and resulting Nyquist plot from fit of data from right side of panel B. (D) Nyquist plots of individual maximally Ca\(^{2+}\)-activated MHC I, IIA and IIB fibers for control and CKD, where each open circle (control) or triangle (CKD) represents one of the oscillation frequencies (1 to 200 Hz) performed during sinusoidal analysis. The sinusoidal analysis results are well-characterized by the continuous (control) or dashed (CKD) lines calculated using the six-parameter model.

Figure 2. Single skeletal muscle fiber maximal Ca\(^{2+}\)-activated specific tension, force and cross-sectional area (CSA) for control, CKD and CKDFE groups by fiber type. Maximal Ca\(^{2+}\)-activated conditions were achieved at pH = 7.0, \(P_i = 5\) mM, pCa = 4.5. Left (A-C): Mean \(\pm\) SE with individual datapoints overlaid on-top of bars. Horizontal bars = significant difference (\(p <\)
0.05) between groups within fiber type. Right (D-F): Scatterplots, with each point representing an individual fiber. Lines indicate linear regressions, with Pearson’s correlation coefficients (r) for Panel D (Control: $r = 0.65$, $p = 0.041$; CKD: $r = 0.69$, $p < 0.001$; CKDFE: $r = 0.77$, $p = 0.004$), E (Control: $r = 0.59$, $p < 0.001$; CKD: $r = 0.51$, $p < 0.001$; CKDFE: $r = 0.50$, $p = 0.004$) and F (Control: $r = 0.81$, CKD: $r = 0.61$, CKDFE: $r = 0.50$; all $p < 0.001$). Number of fibers tested for control/CKD/CKDFE were 10/30/12 for MHC I, 69/59/31 for MHC IIA and 58/67/47 for MHC IIB. Number of mice in each group for control/CKD/CKDFE were 5/5/5. Differences between the groups were assessed by ANOVA including a random effect to account for repeat measurements within mice.

Figure 3. Sinusoidal analysis model for maximal Ca\(^{2+}\)-activated fibers for control, CKD and CKDFE groups by fiber type. Maximal Ca\(^{2+}\)-activated conditions were achieved at pH = 7.0, $P_i = 5$ mM, $pCa = 4.5$. (A) Parameter $B$ is proportional to the number of myosin heads strongly bound to actin and cross-bridge stiffness. (B-C): $A$-elastic and $A$-viscous are the elastic and viscous portions of the myofilament lattice structure and the attached myosin heads. (D) $2\pi b$ is the rate of myosin transition between the weakly and strongly bound states. $E$: $t_{on}$ is the mean time myosin is attached to actin. Mean $\pm$ SE with individual datapoints overlaid on-top of bars. Horizontal bars = significant difference ($p < 0.05$) between groups within fiber type. Number of fibers tested for control/CKD/CKDFE were 10/30/12 for MHC I, 69/59/31 for MHC IIA and 58/67/47 for MHC IIB. Number of mice in each group for control/CKD/CKDFE were 5/5/5. Differences between the groups were assessed by ANOVA including a random effect to account for repeat measurements within mice.
Figure 4. Correlation analysis of the relationship between functional parameters of single muscle fibers and hemoglobin. Mean maximal Ca\textsuperscript{2+}-activated (pH = 7.0, P\textsubscript{i} = 5 mM, pCa = 4.5) specific tension (A-B) and parameter B (C-D) for MHC IIA and IIB fibers, and cross-bridge kinetics, 2\pi b (E) and t\textsubscript{on} (F), for MHC IIA fibers for each mouse plotted against hemoglobin levels. Parameter B is proportional to the number of myosin heads strongly bound to actin and cross-bridge stiffness. 2\pi b is the rate of myosin transition between the weakly and strongly bound states and t\textsubscript{on} is the mean time myosin is attached to actin. Lines indicate linear regressions, with Pearson’s correlation coefficients (r) and respective p-value shown in each panel; n=5 mice per group.

Figure 5. Single skeletal muscle fiber specific tension and dynamic stiffness at various sarcomere lengths under relaxed conditions for control and CKD groups by fiber type. (A-C) Single skeletal muscle fiber specific tension. (D-F) Dynamic stiffness. Relaxed conditions were modeled at pH = 7.0, P\textsubscript{i} = 5 mM, pCa = 8.0 including 2,3-butanedione monoxime (BDM), which placed myosin heads in a weakly bound state. Mean ± SE with individual datapoints offset to the right of mean. † = significant difference (p < 0.05) between groups. Number of fibers tested for control/CKD were 7/7 for MHC I, 22/27 for MHC IIA and 22/30 for MHC IIB. Number of mice in each group for control/CKD was 5/5. Differences between the groups were assessed by ANOVA including a random effect to account for repeat measurements within mice.

Figure 6. Single skeletal muscle fiber maximal Ca\textsuperscript{2+}-activated to rigor dynamic stiffness ratio and maximal Ca\textsuperscript{2+}-activated and rigor dynamic stiffness for control and CKD groups by fiber type. Ca\textsuperscript{2+}-activated conditions were modeled at pH = 7.0, P\textsubscript{i} = 5 mM, pCa = 4.5, ATP = 5 mM and rigor conditions at pH = 7.0, P\textsubscript{i} = 5 mM, pCa = 4.5, ATP = 0 mM. Mean ± SE with
individual datapoints overlaid on-top of bars. Horizontal bars = significant difference ($p < 0.05$) between groups within fiber type. Number of fibers tested for control/CKD were 13/9 for MHC I, 44/35 for MHC IIA and 22/31 for MHC IIB. Number of mice in each group for control/CKD was 5/5. Differences between the groups were assessed by ANOVA including a random effect to account for repeat measurements within mice.

**Figure 7. Specific tension and sinusoidal analysis model parameters for fatigued fibers for control, CKD and CKDFE groups by fiber type.** Fatigued conditions were modeled at pH = 6.2, $P_i = 30$ mM, $pCa = 4.5$. Mean ± SE with individual datapoints overlaid on-top of bars. (A) Specific tension. (B): Parameter $B$ is proportional to the number of myosin heads strongly bound to actin and cross-bridge stiffness. (C-D): $A$-elastic and $A$-viscous are the elastic and viscous portions of the myofilament lattice structure and the attached myosin heads. (E): $2\pi b$ is the rate of myosin transition between the weakly and strongly bound states. (F): $t_{on}$ represents the mean time myosin is attached to actin. Horizontal bars = significant difference ($p < 0.05$) between groups within fiber type, * = significant difference compared to maximal activating condition within group and fiber type. Number of fibers tested for control/CKD/CKDFE for specific tension were 5/19/8 for MHC I, 37/33/21 for MHC IIA and 29/39/31 for MHC IIB and for sinusoidal analysis were 5/19/8 for MHC I, 30/26/20 for MHC IIA and 26/27/31 for MHC IIB. Number of mice in each group for control/CKD/CKDFE were 3/3/3. Differences between the groups were assessed by ANOVA including a random effect to account for repeat measurements within mice.

**Figure 8. Absolute and normalized mean specific tension-pCa relationships for control, CKD and CKDFE groups by fiber type.** Specific tension was assessed at pH = 7.0, $P_i = 5$ mM. Horizontal dashed lines in D-F represent 50% of maximal $Ca^{2+}$-activation, which when crossing
the Hill fit line represents the pCa$_{50}$ value for that group. + = significant difference ($p < 0.05$) between control, CKD and CKDFE, # = significant difference between CKD and CKDFE versus control, ^ = significant difference between control and CKD versus CKDFE. Fibers tested for control/CKD/CKDFE were 5/20/8 for MHC I, 45/37/22 for MHC IIA and 41/56/34 for MHC IIB. Number of mice in each group for control/CKD/CKDFE were 3/3/3. Differences between the groups were assessed by ANOVA including a random effect to account for repeat measurements within mice.
Figure 1

A) Length change (Strain input, $\varepsilon$) → Muscle fiber (Viscoelastic material) → Force Response (Stress output, $\sigma$)

B) $\sigma = \sigma_e + \sigma_v = $ elastic + viscous

Stress response for 15 Hz strain

C) Viscoelastic response

D) Comparison of MHC types

- MHC I
- MHC IIA
- MHC IIB

$\gamma(\omega) = A (i \omega / \alpha) k - B (i \omega / (2 \pi b + i \omega))$

$ + C / (2 \pi c + i \omega)$
Figure 5

(A) MHC I

Specific Tension (N/mm²)

Sarcomere Length (µm)

Control (○)

CKD (▼)

(B) MHC IIA

Specific Tension (N/mm²)

Sarcomere Length (µm)

(C) MHC IIB

Specific Tension (N/mm²)

Sarcomere Length (µm)

(D) Stiffness (N/mm²)

Sarcomere Length (µm)

2.65 µm 4.0 µm

(E) Stiffness (N/mm²)

Sarcomere Length (µm)

(F) Stiffness (N/mm²)

Sarcomere Length (µm)
Supplementary Materials

Supplemental Table 1. Statistical methods used for each experimental condition

Supplemental Figure 1. Body weight during the experimental period

Supplemental Figure 2. Single skeletal muscle fiber force and cross-sectional area (CSA) for control, CKD and CKDFE groups by fiber type and representative gels

Supplemental Figure 3. Assessment of apoptosis in skeletal muscle in three groups of mice
### Supplementary Table 1. Statistical methods used for each experimental condition

<table>
<thead>
<tr>
<th>Statistical Analysis</th>
<th>Purpose</th>
<th>Unit of Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>3X3 Linear Mixed Model Factorial ANOVA</strong></td>
<td>Examine interactions and main effects (mouse condition X fiber condition) for cellular and molecular parameters</td>
<td>Individual fibers, accounting for repeat measurements within mice</td>
</tr>
<tr>
<td>Linear Regression</td>
<td>Relationships between force and CSA</td>
<td>Individual fibers</td>
</tr>
<tr>
<td></td>
<td>Relationships between specific tension, $B$, and cross-bridge kinetics with hemoglobin</td>
<td>Individual mice</td>
</tr>
<tr>
<td>ANCOVA</td>
<td>Examine mean differences between slopes and intercepts from linear regression</td>
<td>Individual fibers</td>
</tr>
<tr>
<td><strong>Linear Mixed Model ANOVA</strong></td>
<td>Examine differences in calcium sensitivity and specific tension-pCa experiments utilizing Bonferroni post-hoc tests for mean differences</td>
<td>Individual fibers, accounting for repeat measurements within mice</td>
</tr>
<tr>
<td></td>
<td>Determine significance of the differences in physical parameters, kidney function and hemoglobin between experimental groups utilizing Bonferroni post-hoc tests for mean differences</td>
<td>Individual fibers, accounting for repeat measurements within mice</td>
</tr>
<tr>
<td></td>
<td>Examine mean differences for passive stiffness experiments, rigor experiments, and relative changes from maximal $Ca^{2+}$-activation to fatigue</td>
<td>Individual fibers, accounting for repeat measurements within mice</td>
</tr>
</tbody>
</table>

CSA = Cross-sectional area
Supplemental Figure 1
Supplemental Figure 2
Supplemental Figure 3
**Supplemental Figure 1. Body weight during the experimental period.** Mice were started on control diet or 0.2% adenine diet at 8 weeks of age and euthanized at 16 weeks of age. + = significant difference \((p < 0.05)\) within each group from initial body weight at time zero (8 weeks of age), # = significant difference between CKD and CKDFE versus control at each time point. Number of mice in each group for control/CKD/CKDFE were 10/12/6.

**Supplemental Figure 2. Single skeletal muscle fiber force and cross-sectional area (CSA) for control, CKD and CKDFE groups by fiber type and representative gels.** Mean ± SE with individual datapoints overlaid on-top of bars for fatiguing conditions \((\text{pH} = 6.2, \text{P}_i = 30 \text{ mM})\). Horizontal bars = significant difference \((p < 0.05)\) between groups within fiber type, * = significant difference compared to maximal activating condition within group. Right \((D-F)\): Scatterplots, with each point representing an individual fiber. Lines indicate linear regressions, with Pearson’s correlation coefficients for Panel C (Control: \(r = 0.64, p = 0.24\), CKD: \(r = 0.49, p = 0.035\), and CKDFE: \(r = 0.91, p = 0.001\)), D (Control: \(r = 0.22, p = 0.18\), CKD: \(r = 0.18, p = 0.30\), and CKDFE: \(r = 0.68, p = 0.001\)), and E (Control: \(r = 0.63, p < 0.001\), CKD: \(r = 0.32, p = 0.049\), and CKDFE: \(r = 0.42, p = 0.02\)). Number of mice in each group for control/CKD/CKDFE were 3/3/3.

**Supplemental Figure 3. Assessment of apoptosis in skeletal muscle in three groups of mice.** Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining of gastrocnemius muscle for control, CKD, and CKDFE groups was negative. Representative images are shown; images from \(n = 3\) mice per group were reviewed.