Senescence Is Associated With Elevated Intracellular Resting \([\text{Ca}^{2+}]\) in Mice Skeletal Muscle Fibers. An *in vivo* Study

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Aging causes skeletal muscles to become atrophied, weak, and easily fatigued. Here, we have tested the hypothesis that normal aging in skeletal muscle cells is associated with \(\text{Ca}^{2+}\) intracellular dyshomeostasis and oxidative stress. Intracellular \(\text{Ca}^{2+}\) concentration \((\text{[Ca}^{2+}]_i)\), resting intracellular \(\text{Na}^+\) concentration \((\text{[Na}^+]_i)\) and reactive oxygen species (ROS) production were measured *in vivo* (superficial gastrocnemius fibers) using double-barreled ion-selective microelectrodes, and *in vitro* (isolated single flexor digitorum brevis fibers) using fluorescent ROS sensor CM-H2DCFDA in young (3 months of age), middle-aged (12 months of age), and aged (24 months of age) mice. We found an age-related increase in \([\text{Ca}^{2+}]_i\) from 121 ± 4 nM in young muscle cells which rose to 255 ± 36 nM in middle-aged and to 409 ± 25 nM in aged cells. \([\text{Na}^+]_i\) also showed an age-dependent elevation, increasing from 8 ± 0.5 mM in young muscle fibers, to 12 ± 1 mM in middle-aged and to 17 ± 1 mM in old muscle fibers. Using the fluorescent ROS sensor CM-H2DCFDA we found that these increases in intracellular cation concentrations were associated with significantly increased basal ROS production as demonstrated by age related increases in the rate of dichlorodihydrofluorescein fluorescence. To determine is this could be modified by reducing ROS and/or blocking sarcolemmal \(\text{Ca}^{2+}\) influx we administered flufenamic acid (FFA), a non-steroidal anti-inflammatory drug which is also a non-selective blocker of the transient receptor potential canonical channels (TRPCs), for 4 weeks to determine if this would have a beneficial effect. FFA treatment reduced both basal ROS production and muscle \([\text{Ca}^{2+}]_i\) and \([\text{Na}^+]_i\) in middle-aged and aged muscle fibers compared to fibers and muscles of untreated 12 and 24-months old mice. \([\text{Ca}^{2+}]_i\) was reduced to 134 ± 8 nM in middle-aged muscle and to 246 ± 40 nM in muscle from aged mice. Likewise \([\text{Na}^+]_i\) was reduced to 9 ± 0.7 mM in middle-aged muscles and to 13 ± 1 mM in muscle from aged mice. FFA treatment also reduced age associated increases in plasma interleukin 6 and tumor necrosis factor-alpha (TNF-α) concentrations which were elevated in 12 and 24-months old mice compared to young mice and decreased age-related muscle damage as indicated by a reduction in serum creatine kinase (CK).
**INTRODUCTION**

Aging is associated with a concomitant reduction in skeletal mass and muscle strength with a lack of causal disease (Rolland et al., 2008; Tieland et al., 2018). The etiology of muscle aging is complex and still is not fully elucidated. Diverse alterations in muscle have been described, e.g., a significant decrease in myofiber size and number, improper protein synthesis–degradation, and a reduction in the magnitude of motor neurons innervating muscle cells (Doherty, 2003; Mosole et al., 2014). The reduction in muscle mass and strength observed during senescence compromises physical activity promoting a sedentary lifestyle that predisposes the individual to the risk of falling and even death (Visser and Schaap, 2011). Dysfunctional excitation–contraction coupling (Delbono et al., 1995), reduced density of calcium release units (Boncompagni et al., 2006), increased oxidative stress, and alterations of mitochondrial function appears to play a role in age-related changes in muscle (Pietrangelo et al., 2015).

Although abnormalities in intracellular calcium regulatory mechanisms in skeletal muscle with aging have been suggested, no systematic study has been conducted. To our knowledge, the present study represents the first attempt to determine the impact of senescence on intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) in skeletal muscle, and the role of Ca$^{2+}$ influx mediated by the transient receptor potential canonical (TRPC channels) observed with aging. In addition, we also probed the contribution of oxidative stress and the inflammatory cytokines interleukin (IL-6) and tumor necrosis factor-alpha (TNF-$\alpha$). We hypothesized that senescence is associated with a chronic increase in [Ca$^{2+}$]$_i$ and resting intracellular Na$^+$ ([Na$^+$]$_i$) in skeletal muscle cells that could be modified or normalized with pharmacological intervention with flufenamic acid (FFA—an anti-inflammatory and non-selective blocker of TRPC channels) injected intraperitoneally (IP) (12.5 mg/kg) once per day for 4 weeks and in vivo intracellular Ca$^{2+}$ and Na$^+$ determinations were carried out at the age of 12-months. (D) Experimental group 3: 92-week-old mice ($N = 5$) were treated with FFA for 4 weeks, and in vivo intracellular Ca$^{2+}$ and Na$^+$ determinations were carried out at the age of 24-months.

The first cohort of mice was used for in vivo intracellular ion measurements in muscle and consisted of five groups of mice: (A) Control group: Mice ($N = 6$) that did not receive any treatment for the 24 months study and had in vivo intracellular Ca$^{2+}$ and Na$^+$ determinations performed serially at 3, 12, and 24 months. (B) Experimental group 1: 8-week old mice ($N = 7$) were treated for 4 weeks with flufenamic acid (FFA—an anti-inflammatory and non-selective blocker of TRPC channels) injected intraperitoneally (IP) (12.5 mg/kg) once per day for 4 weeks and in vivo intracellular Ca$^{2+}$ and Na$^+$ determinations were carried out at the age of 3-months. (C) Experimental group 2: 44-week old mice ($N = 6$) were treated with FFA for 4 weeks, and in vivo intracellular Ca$^{2+}$ and Na$^+$ determinations were carried out at the age of 12-months. (D) Experimental group 3: 92-week-old mice ($N = 5$) were treated with FFA for 4 weeks, and in vivo intracellular Ca$^{2+}$ and Na$^+$ determinations were carried out at the age of 24-months.

The second cohort of 24 mice were randomly divided into six groups (three treatment and three control, $N = 4$ mice per group) and given the same treatment that was used in the first cohort at 8, 44, and 92 weeks and the studies carried out at 3, 12, and 24 months of age. In this cohort, enzymatically dissociated isolated single flexor digitorum brevis (FDB) muscle cells were used to determine ROS production rate using a fluorescence assay. Unlike the first group, where it was possible to serial measurements in the control group, separate control groups were used for each time point.

The third cohort of 36 mice of each age were randomly divided into six groups (three treatment and three controls, $N = 6$ mice per group) and given the same treatment given as in the first cohort at 8, 44, and 92 weeks and the studies carried out at 3-, 12- and 24-months of age. At that time, the mice blood was collected from the tail vein for measurements of plasma IL-6, TNF-$\alpha$, and CK concentrations, and the animals were then euthanized by cervical dislocation.

**MATERIALS AND METHODS**

**Animals and Experimental Groups**

Young – 3 months; middle-aged – 12 months, and aged – 24 months old C57BL/6J male mice were maintained in the Mount Sinai Medical Center, United States vivarium under constant temperature (21–22°C) on a 12-h light/12-h dark cycle with free access to food and water. All experimental procedures were approved by the Institutional Animal Care and Use Committee at Mount Sinai Hospital.

Activity. Our data provides a direct demonstration that normal aging is associated with a significant elevation [Ca$^{2+}$], [Na$^+$], and intracellular ROS production in skeletal muscle fibers. Furthermore, the fact that FFA reduced the intracellular [Ca$^{2+}$], [Na$^+$], and ROS production as well as the elevated IL6, TNF-$\alpha$, and CK levels, led us to suggest that its pharmacological effect may be related to its action both as a TRPC channel blocker and as an anti-inflammatory.

**Keywords:** aging, calcium, TRPC, skeletal muscle, inflammation

**Ca$^{2+}$- and Na$^+$-ion Selective Microelectrodes**

Double-barreled, Ca$^{2+}$- or Na$^+$-selective microelectrodes were prepared from thin-walled 1.2- and 1.5-mm outside diameter (OD) borosilicate HCl-washed glass capillaries (PB150F-4,
World Precision Instruments, FL, United States). They were heat sterilized (UX-10776-00, Cole Palmer, IL, United States) stretched in a pipette puller (P-97 Flaming/Brown, Automate Scientific, Berkeley, CA, United States) to obtain small-tipped microelectrodes (<1 μM). The ions-selective barrel (1.5-mm OD) was silanized by exposing it to dimethyldichlorosilane vapor. The tip was then back-filled with either the Ca$^{2+}$ ionophore II (ETH 129, Sigma-Aldrich, MO, United States) or Na$^+$ ionophore (ETH 227, Sigma-Aldrich, MO, United States). The barrel’s remainder was back-filled with pCa7 for the Ca$^{2+}$ selective microelectrode or 8 mM NaCl solution for the Na$^+$ selective as described previously (Eltit et al., 2013). The membrane potential barrel (1.2-mm OD) was back-filled with 3 M KCl just before the measurements were performed. The tip resistances were measured by passing a current pulse of 1 pA through an individual barrel while the electrode tip was in the Ringer bathing solution (Eltit et al., 2013). For the Ca$^{2+}$ and Na$^+$-selective microelectrodes, the resistances ranged from 8 to $10 \times 10^{10}$ Ω, and for the membrane potential microelectrodes (Vm), the resistances ranged from 10 to 15 MΩ with a tip potential less than 5 mV. The double-barreled ion-selective microelectrode was mounted in a modified plastic holder containing Ag/AgCl wires. It was attached to a miniature head stage (probe input impedance $> 10^{13}$Ω), which was connected to a Duo 773 electrometer (World Precision Instruments, FL, United States). The Vm and ions specific potentials were acquired at a frequency of 1,000 Hz with AxoGraph software (version 4.6; Axon Instruments, CA, United States) and stored in a computer for further analysis. Each ion-selective microelectrode was individually calibrated before and after the measurement by exposure of the tip to a series of calibrating solutions, as described previously, and if the two calibration curves did not agree within 3 mV, data from that microelectrode were discarded (Lopez et al., 1983; Eltit et al., 2013). The bath’s reference electrode was either an Ag–AgCl pellet or an agar bridge made of a polyethylene tube containing 3 M KCl gelled in agar. Muscle cells were impaled with the double-barreled ion-microelectrode and Vm and Ca$^{2+}$ or Vm and Na$^+$ potentials were measured. After both potentials were stable for at least 1 min, the microelectrode was withdrawn.

**Recording of Intracellular [Ca$^{2+}$] and [Na$^+$] in Muscle Fibers in vivo**

Intracellular Ca$^{2+}$ and Na$^+$ determinations were carried out in vivo using Ca$^{2+}$- and Na$^+$-selective microelectrodes (Elit et al., 2013) under anesthetic conditions. After the mice were anesthetized, the hair on the leg was removed, the skin was cleaned with an antisepic solution, and a small incision was made. The gastrocnemius muscle was identified, the muscle fascia was partially removed, and the superficial fibers were exposed. Warm sterile Ringer’s solution was perfused onto the superficial muscle fibers to preserve moisture. The mice were kept euthermic (37°C) with the aid of a low noise heating system (ATC1000, World Precision Instruments, FL, United States). Through the aid of a stereomicroscope (233445 Olympus, MA, United States), individual muscle fibers were impaled with either Ca$^{2+}$ or Na$^+$ double-barreled microelectrodes. Examples of actual recordings from these electrodes can be found in our previous publications (Lopez et al., 1983, 2000, 2011, 2020).

In the control group, [Ca$^{2+}$], and [Na$^+$], measurements were repeated in the same animal at all three-time points (3, 12, and 24-months). In this group, after the measurements of [Ca$^{2+}$], and [Na$^+$], were made, the area around the wound was washed with streptomycin solution (10 mg/L), and the skin was sutured. Topical local anesthetic (bupivacaine) was applied every 12 h for 48 h to the wound area to relieve minor pain (type A – wound suturing) produced by survival surgery according to the Guide for the Care and Use of Laboratory Animals. In the treated groups and the control group at 24 months, after completing measurements of [Ca$^{2+}$]i and [Na$^+$]i, mice were euthanized by cervical dislocation. Before making measurements, all mice were kept in individual cages to avoid potential damage by other mice.

**Determination of Reactive Oxygen Species in Muscle Cells**

Intracellular ROS levels were determined in enzymatically isolated FDB muscle cells from treated and untreated 3, 12, and 24-month-old mice using the dichlorodihydrofluorescein diacetate (DCFHDA) assay (Luis, MO, United States) as previously described (Lopez et al., 2018b). The fluorescence intensity of dichlorodihydrofluorescein (DCF) was detected by a fluorescence microplate reader (Molecular Device, Sunnyvale, CA, United States) at an excitation wavelength of 488 nm and an emission wavelength 525 nm. All measurements were performed in triplicate, and the results reported as the percentage of ROS production relative to untreated 3-month muscle cells.

**Determination of Plasma IL6 and TNF-α Concentrations and CK Activity**

Plasma IL-6 concentrations were measured in plasma samples from blood collected from the tail vein in treated and untreated 3, 12, and 24 months-old animals using a Milliplex Mouse Cytokine/Chemokine Panel (EMD Millipore, MA, United States) with a Bio-Plex Suspension Array System (Bio-Rad Laboratories, CA, United States). Similarly, TNF-α content was measured in plasma samples from untreated and treated 3, 12, and 24 months-old animals using a mouse TNF-α ELISA kit (Invitrogen, CA, United States). The values were normalized to muscle protein concentration determined by BCA protein assay (Thermo Scientific, MA, United States). Plasma CK activity was determined using a creatine kinase assay kit (Sekisui Diagnostics, MA, United States) according to manufacturer’s instructions. CK activity was determined in triplicate from each sample and expressed as units per liter (U/L). All measurements were carried out on triplicate blood samples obtained from the same animal at three different times on the same day (8 am, 12 pm, and 4 pm). Despite the small blood volume withdrawn, oral fluid replacement was provided between each endpoint.

**Statistics**

Data are reported as mean ± standard deviation (SD). For [Ca$^{2+}$]i and [Na$^+$]i, determinations, each successful impalement
was considered one experimental n. We excluded all data from muscle fibers showing a resting membrane potential of less than $-80$ mV from the final analysis (22% of the total fibers measured). In the biochemical assay, each animal represented one experimental N and n the number of measurements. We used the D’Agostino and Pearson test to determine whether the samples were normally distributed. We compared the experimental values using a one-way analysis of variance (ANOVA) and Tukey post hoc test. A p-value $<0.05$ was set as statistically significant level. All statistical analyses were carried out with GraphPad Prism 9.0 (GraphPad Software, CA, United States).

RESULTS

Muscle Intracellular $[\text{Ca}^{2+}]_i$ and $[\text{Na}^+]_i$ and Aging. Effects of FFA

We examined muscle $[\text{Ca}^{2+}]_i$ and $[\text{Na}^+]_i$, in vivo in young, middle-aged, and aged mice to explore the changes associated with normal aging in mice. Compared to young muscle fibers $[\text{Ca}^{2+}]_i$, from middle-aged and aged mice was significantly elevated. In young mice, muscle $[\text{Ca}^{2+}]_i$ was $121 \pm 4$ nM ($N = 6, n = 15$). In middle-aged mice muscle $[\text{Ca}^{2+}]_i$, increased to $255 \pm 36$ nM ($N = 6, n = 16, p \leq 0.001$ compared to young mice), and in aged mice it rose to $409 \pm 35$ nM ($N = 6, n = 14, p \leq 0.001$ compared to young mice; Figure 1 left panel). We also found that muscle $[\text{Na}^+]_i$ is also significantly elevated in middle-aged and aged muscle compared to young mice. In muscle fibers from young mice $[\text{Na}^+]_i$, was $8 \pm 0.5$ mM, ($N = 6, n = 11$), from middle-aged mice $[\text{Na}^+]_i$, was $12 \pm 1$ mM ($N = 6, n = 13, p \leq 0.001$ compared to young mice) and from aged mice $[\text{Na}^+]_i$, was $17 \pm 1$ mM ($N = 6, n = 12, p \leq 0.001$ compared to young mice; Figure 2 left panel).

Treatment with FFA (12.5 mg/kg IP once per day for 4 weeks; see section “Materials and Methods” for more details) normalized $[\text{Ca}^{2+}]_i$ in muscle fibers from middle-aged mice (134 ± 8 nM, $N = 6, n = 16, p \geq 0.65$ compared to young muscle cells) and significantly reduced $[\text{Ca}^{2+}]_i$ in muscle cells from aged mice (246 ± 40 nM, $N = 5, n = 18, p \leq 0.001$ compared to untreated aged mice). No effect of FFA treatment on $[\text{Ca}^{2+}]_i$ was observed in muscle fibers from young mice (Figure 1 right panel). Similarly, FFA treatment normalized $[\text{Na}^+]_i$ in muscle cells from middle-aged mice (9 ± 0.7 mM, $N = 6, n = 11, p \geq 0.52$ compared to young muscle fibers) and significantly reduced $[\text{Na}^+]_i$ in muscle from aged mice (13 ± 1 mM, $N = 5, n = 12, p \leq 0.001$ compared to muscle from untreated aged mice). FFA did not modify $[\text{Na}^+]_i$ in young muscle cells (Figure 2 right panel).

Increased Oxidative Stress With Aging

We measured ROS production in isolated FDB muscle cells from young, middle-aged, and aged mice. FDB muscle fibers loaded with the fluorescent ROS sensor CM-H2DCFDA in middle-aged and aged mice showed significantly increased rates of DCF fluorescence increase compared to FDB muscle fibers isolated from young mice ($p \leq 0.001$ for both middle-aged and aged fibers compared to muscle from young mice; Figure 3 left panel). Evidence has been presented suggesting that $[\text{Ca}^{2+}]_i$ could modulate either ROS production and/or clearance in excitable cells (Gorlach et al., 2015). Thus, we reasoned that reducing muscle $[\text{Ca}^{2+}]_i$ by treatment with FFA (see Figure 1 right panel) should decrease intracellular ROS production. Pre-treatment with FFA (12.5 mg/kg IP once per day for four weeks) significantly reduced ROS production in middle-aged and aged muscle cells ($p \leq 0.001$ compared to muscles from untreated middle-aged and aged mice; Figure 3 right panel). No effect of FFA treatment on ROS production was detected in muscle cells from young mice ($p \geq 0.98$ compared to muscle cells from untreated mice; Figure 3 right panel).

Plasma IL-6 and TNF-α Concentrations and Aging

Senescence has been shown to be associated with an increase in inflammatory markers such as IL6 and TNF-α (Wei et al., 1992; Garner et al., 2018). Therefore, we measured IL-6 levels and TNF-α levels in plasma from young, middle-aged, and aged mice. We found that plasma IL-6 concentration increased
from 19 ± 2 pg/mL (N = 6 mice, n = 13) in young mice, to 30 ± 3 pg/mL in middle-aged mice (N = 6 mice, n = 14, p < 0.001 compared to young mice), and to 50 ± 6 pg/mL in aged mice (N = 6 mice, n = 16, p < 0.001 compared to young mice). (Figure 4 left panel). Similarly, we found that there was an age-dependent elevation of plasma TNF-α concentration from 5.9 ± 1.1 pg/mg protein (N = 6 mice, n = 15) in young mice to 9.6 ± 1.6 pg/mg protein middle-aged mice (N = 6 mice, n = 16, p < 0.001 compared to young mice) and to 15.9 ± 2.1 pg/mg protein aged mice (N = 6 mice, n = 18, p < 0.001 compared to young mice; Figure 5 left panel). Treatment with FFA (12.5 mg/kg IP once per day for four weeks) normalized plasma IL-6 levels in middle-aged mice (22 ± 3 pg/mL, N = 6 mice, n = 16, p ≥ 0.31 compared to plasma levels from young mice and p ≥ 0.001 compared to plasma IL-6 levels from untreated middle-aged mice). In aged mice FFA reduced plasma IL-6 levels to 36 ± 4 pg/mL (N = 6 mice, n = 15, p < 0.001 compared to plasma IL-6 levels from untreated aged mice; Figure 4 right panel). Likewise FFA treatment significantly reduced TNF-α levels in both middle-aged mice group where it was normalized to the levels of untreated young mice (6 ± 1.3 pg/mg protein, N = 6 mice, n = 15, p ≥ 0.99 compared to plasma levels from young mice) and in the aged group it was reduced to 10.7 ± 1.9 pg/mg protein, N = 6 mice, n = 18, p < 0.001 compared to plasma concentrations from untreated aged-mice; Figure 5 right panel) In young mice, FFA did not change the IL-6 plasma level (20 ± 3 pg/mL, N = 6 mice, n = 14, p ≥ 0.99 compared to plasma IL-6 levels from untreated young mice; Figure 4 right panel) or TNF-α levels (5.6 ± 1.2 pg/mg protein, N = 6 mice, n = 16, p ≥ 0.98 compared to plasma concentration from untreated aged-mice; Figure 5 right panel).

**Muscle Damage and Aging**

Although an increase in plasma CK can indicate acute cardiac muscle damage, levels which remain increased over time are more likely to be an indication of skeletal muscle damage which has previously been shown to be associated with aging (Kim et al., 2018). Here we found that plasma CK activity was significantly elevated in both middle-aged mice (141 ± 13 IU/L,
DISCUSSION

In this study we directly examined changes in resting intracellular Ca\(^{2+}\) and Na\(^{+}\) homeostasis in skeletal muscle during normal aging. The major findings of the present study are an age-dependent increase of skeletal muscle [Ca\(^{2+}\)], [Na\(^{+}\)], increased ROS generation and elevation of plasma IL-6, TNF-\(\alpha\) and CK concentrations (aged > middle-aged > young). Treatment with FFA, reduced muscle levels of [Ca\(^{2+}\)], [Na\(^{+}\)], reduced ROS production and reduced plasma levels of IL-6, TNF-\(\alpha\) and CK in middle aged and aged mice (12 and 24-months) but had no effect in young mice (3-months).

Regulation of resting intracellular calcium [Ca\(^{2+}\)] is critical in muscle cells. While extracellular Ca\(^{2+}\) concentration ranges within 1–2 mM in quiescent and healthy muscle cells resting free cytosolic [Ca\(^{2+}\)] is remarkably low (10\(^{-7}\) M) (Marban et al., 1980; Lopez et al., 1983, 2018a). Several intracellular regulatory mechanisms have been proposed in muscle cells to preserve this low resting [Ca\(^{2+}\)]. ATP-driven Ca\(^{2+}\) pumps pump out Ca\(^{2+}\) from the cytoplasm, including the sarcoplasmic reticulum (SR) Ca\(^{2+}\)-ATPase that pumps it back into the SR and the plasma membrane Ca\(^{2+}\)-ATPase (Brini and Carafoli, 2009) that pumps it out of the cytoplasm into the extracellular space. Furthermore [Ca\(^{2+}\)]\(_i\) is also regulated by Ca\(^{2+}\) influx...
through TRPC channels (Choi et al., 2020), store-operated Ca\(^{2+}\) channels (Lyfenko and Dirksen, 2008), the bidirectional and electrogenic Na\(^{+}\)/Ca\(^{2+}\) exchanger in the plasma membrane (Cifuentes et al., 2000; Fraysse et al., 2001; Blaustein, 2013) and by passive leak from the SR through the ryanodine receptor (Yang et al., 2007; Eltit et al., 2010; Andersson et al., 2011; Lamboley et al., 2016). This leak then provokes a chronic partial depletion of SR Ca\(^{2+}\) which induces an increased influx of extracellular Ca\(^{2+}\) that is independent of the L-type Ca\(^{2+}\) channel mediated Ca\(^{2+}\) entry (Laumikonis and Rios, 2007; Lyfenko and Dirksen, 2008). This pathway's identity is not fully understood; however, the involvement of TRPCs, which are highly expressed in skeletal muscle cells, has been suggested (Vandebrouck et al., 2002; Millay et al., 2009). A similar aberrant elevation in [Ca\(^{2+}\)]\(_i\) and [Na\(^{+}\)]\(_i\) have been observed in muscle cells from patients with Duchenne's muscular dystrophy and mdx mice (Lopez et al., 1987; Altamirano et al., 2014b; Lopez et al., 2017, 2020) where an abnormal Ca\(^{2+}\) influx has also been reported (Tutdibi et al., 1999; Kruger et al., 2008; Millay et al., 2009). Thus, if it were possible to prevent this chronic elevation of [Ca\(^{2+}\)]\(_i\) during aging, it may exert a myo-protective effect and potentially prevent the muscle wasting and dysfunction observed in the aging muscle.

We show here that there is a significant increase in ROS generation in aging muscles (old > middle-aged > young). Physiological concentrations of ROS play essential roles in diverse muscle signaling pathways. However, elevated ROS production harms muscle function (Wanagat et al., 2001; Calvani et al., 2013; Le Moal et al., 2017). ROS and lipid peroxidation levels are abnormal in senescent muscle cells (Ryan et al., 2010). However, there is a large body of evidence showing mitochondrial dysfunction is associated with aging, and it is well known that the mitochondria are a primary source of cells ROS production (Boengler et al., 2017). Intracellular ROS dyshomeostasis has been suggested to decrease protein synthesis and increase protein degradation, provoking muscle atrophy (Kinugawa et al., 2015) and microdamage of the muscle membrane allowing release of intracellular components such as CK (Kim et al., 2018). One of the results of increased ROS production is an increase in the plasma levels inflammatory markers such as IL-6 and TNF-α such as we found here. The above-described results suggest that increased ROS production in senescent skeletal muscle might be linked with age-dependent intracellular Ca\(^{2+}\) imbalance observed in aging mice (Kinugawa et al., 2015).

To further explore the mechanism involved in the changes of [Ca\(^{2+}\)]\(_i\), [Na\(^{+}\)]\(_i\), ROS, IL-6, TNF-α with aging we treated mice
with FFA, a NSAID drug which also blocks TRPCs which are non-selective plasmalemmal cation channels (Chen et al., 1993; Hescheler and Schultz, 1993). The FFA anti-inflammatory effect appears to be mediated by reduction of prostaglandin synthesis by inhibiting the cyclo-oxygenases (Flower, 1974). Clinically FFA has been used locally for analgesia against pain and inflammation associated with musculoskeletal and joint disorders, peri-articular, and soft tissue disorders (Flower, 1974). In addition, FFA has been used as a non-selective plasmalemmal cation entry channel blocker and has been shown to inhibit the spontaneous active tone of carotid artery (Shimamura et al., 2002) and to attenuate the K+ induction in endothelium-denuded small and large arteries (Bencze et al., 2015). In this study, we demonstrated that administration of FFA reduced intracellular Ca2+ and Na+ overload, decreased the rate of ROS production, and lowered the high plasma concentration levels of IL-6 and TNF-α and CK activity in aging mice. Due to FFA's lack of pharmacological specificity (anti-inflammatory and TRPC channel blocker), we are unable to dissect which of these is the primary mechanism of action and which is the result of the primary action.

**CONCLUSION**

In this study we present direct evidence of abnormal regulation of [Ca2+], [Na+], and increased ROS production in aging muscles. Furthermore, we show that aging is associated with elevated plasma levels of the inflammatory markers, IL6, TNF-α, and CK which is an indicator of chronic muscle damage. Treatment with FFA significantly decreased elevated [Ca2+], [Na+], and reduced ROS overload, which was accompanied by decreases in plasma IL6, TNF-α, and CK levels. The mechanism of FFA's action in correcting these age related defects may be related either to its action as a TRPC channel blocker and/or its direct anti-inflammatory effect on ROS production.

**DATA AVAILABILITY STATEMENT**

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

**ETHICS STATEMENT**

All protocols used in the study were performed following the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and approved by the IACUC of the Mount Sinai Medical Center, United States.

**AUTHOR CONTRIBUTIONS**

AM and JL performed the research and analyzed the data. AM, JL, and PA wrote the manuscript. All authors contributed to the manuscript revision and read and approved the submitted version.

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**REFERENCES**

Altamirano, F., Elitti, J. M., Robin, G., Linares, N., Ding, X., Pessah, I. N., et al. (2014a). Ca2+ influx via the Na+/Ca2+ exchanger is enhanced in malignant hyperthermia skeletal muscle. J Biol Chem 289, 19180–19190. doi: 10.1074/jbc.m114.550764

Altamirano, F., Perez, C. F., Liu, M., Widrick, J., Barton, E. R., Allen, P. D., et al. (2014b). Whole body periodic acceleration is an effective therapy to ameliorate muscle weakness in mdx mice. PLoS One 9:e106590. doi: 10.1371/journal.pone.0106590

Andersson, D. C., Betzenhauser, M. J., Reiken, S., Meli, A. C., Umsanskaya, A., Xie, W., et al. (2011). Ryanodine receptor oxidation causes intracellular calcium leak and muscle weakness in aging. Cell Metab 14, 196–207. doi: 10.1016/j.cmet.2011.05.014

Bencze, M., Behuliak, M., Vavrinova, A., and Zicha, J. (2015). Broad-range TRP channel inhibitors (2-APB, flufenamic acid, SKF-96365) affect differently contraction of resistance and conduit femoral arteries of rat. Eur J Pharmacol 765, 533–540. doi: 10.1016/j.ejphar.2015.09.014

Blaustein, M. P. (2013). Livin’ with NCX and lovin’ it: a 45 year romance. Adv Exp Med Biol 961, 3–15. doi: 10.1007/978-1-4614-4756-6_1

Boengler, K., Kosiol, M., May, T., Schulz, R., and Rohrbach, S. (2017). Mitochondria and ageing: role in heart, skeletal muscle and adipose tissue. J Cell Physiol 232, 1994–2004. doi: 10.1002/jcp.25960

Boncompagni, S., D’amelio, L., Fulle, S., Fano, G., and Protasi, F. (2006). Progressive disorganization of the excitation-contraction coupling apparatus in aging human skeletal muscle as revealed by electron microscopy: a possible role in the decline of muscle performance. J Gerontol A Biol Sci Med Sci 61, 995–1008. doi: 10.1093/gerona/61.10.995

Brini, M., and Carafoli, E. (2009). Calcium pumps in health and disease. Physiol Rev 89, 1341–1378. doi: 10.1152/physrev.00032.2008

Burr, A. R., Millay, D. P., Goonasekera, S. A., Park, K. H., Sargent, M. A., Collins, J., et al. (2014). Na+ dysregulation coupled with Ca2+ entry through NCX1 promotes muscular dystrophy in mice. Mol Cell Biol 34, 1991–2002. doi: 10.1128/mcb.00339-14

Calvani, R., Joseph, A. M., Adhibetty, P. J., Miccheli, A., Bossola, M., Leeuwenburgh, C., et al. (2013). Mitochondrial pathways in sarcopenia of aging and disuse muscle atrophy. Biol Chem 394, 393–414. doi: 10.1515/bch-2012-0247

Chen, S., Inoue, R., and Ito, Y. (1993). Pharmacological characterization of muscarinic receptor-activated cation channels in guinea-pig ileum. Br J Pharmacol 109, 793–801. doi: 10.1111/j.1476-5381.1993.tb13644.x

Choi, J. H., Jeong, S. Y., Oh, M. R., Allen, P. D., and Lee, E. H. (2020). TRPCs: Influential Mediators in Skeletal Muscle. Cells 9, 850. doi: 10.3390/cells9040850

Cifuentes, F., Vargara, J., and Hidalgo, C. (2000). Sodium/calcium exchange in amphibian skeletal muscle fibers and isolated transverse tubules. Am J Physiol Cell Physiol 279, C89–C97

Delbono, O., O’rourke, K. S., and Ettinger, W. H. (1995). Excitation–calcium release uncoupling in aged single human skeletal muscle fibers. J Membr Biol 148, 211–222.
Doherty, T. J. (2003). Invited review: Aging and sarcopenia. *J Appl Physiol* (1985) 95, 1717–1727. doi: 10.1152/japplphysiol.00347.2003

Eltit, J. M., Ding, X., Pessah, I. N., Allen, P. D., and Lopez, J. R. (2013). Nonspecific sarcocellular cation channels are critical for the pathogenesis of malignant hyperthermia. *FASEB J* 27, 991–1000. doi: 10.1096/fj.12-218354

Eltit, J. M., Yang, T., Li, H., Molinski, T. F., Pessah, I. N., Allen, P. D., et al. (2010). RyR1-mediated Ca2+ leak and Ca2+ entry determine resting intracellular Ca2+ in skeletal myotubes. *J Biol Chem* 285, 13781–13787. doi: 10.1074/jbc.m110.107300

Flower, R. J. (1974). Drugs which inhibit prostaglandin biosynthesis. *Pharmacol Rev* 26, 33–67.

Foster, R. R., Zadeh, M. A., Welsh, G. I., Satchell, S. C., Ye, Y., Mathieson, P. W., et al. (2009). Flufenamic acid is a tool for investigating TRPC6-mediated calcium signalling in human conditionally immortalised podocytes and HEK293 cells. *Cell Calcium* 45, 384–390. doi: 10.1016/j.ceca.2009.01.003

Frayssé, B., Rouaud, T., Millour, M., Fontaine-Perus, J., Gardahaut, M. F., and Levitsky, D. O. (2001). Expression of the Na(+)/Ca(2+) exchanger in skeletal muscle. *Am J Physiol Cell Physiol* 280, C146–C154.

Gaily, P. (2012). TRP channels in normal and dystrophic skeletal muscle. *Curr Opin Pharmacol* 12, 326–334. doi: 10.1016/j.coph.2012.01.018

Garner, K. M., Amin, R., Johnson, R. W., Scarlett, E. J., and Burton, M. D. (2018). Microglia priming by interleukin-6 signaling is enhanced in aged mice. *J Neuroimmunol* 324, 90–99. doi: 10.1016/j.jneuroim.2018.09.002

Gorlach, A., Bertram, K., Hudecova, S., and Krizanova, O. (2015). Calcium and RQS: A mutual interplay. *Redox Biol* 6, 260–271. doi: 10.1016/j.redox.2015.02.004

Hescherle, J., and Schultz, G. (1993). Noneselective cation channels: physiological and pharmacological modulations of channel activity. *EXS* 66, 27–43. doi: 10.1007/978-3-0348-7327-7_2

Jiang, H., Zeng, B., Chen, G. L., Bot, D., Eastmond, S., Elensussi, S. E., et al. (2012). Effect of non-steroidal anti-inflammatory drugs and new fenamate analogues on TRPC4 and TRPC5 channels. *Biochem Pharmacol* 83, 923–931. doi: 10.1016/j.bcp.2012.01.014

Kim, K. Y., Ku, S. K., Lee, K. W., Song, C. H., and An, W. G. (2018). Muscle-protective effects of Schisandrae Fructus extracts in old mice after chronic forced exercise. *Ethnopharmacol* 212, 175–187. doi: 10.1016/j.ethnopharmacol.2017.10.022

Kinugawa, S., Takada, S., Matsuhasha, S., Okita, K., and Tsutsui, H. (2015). Skeletal Muscle Abnormalities in Heart Failure. *Int Heart J* 56, 475–484. doi: 10.1536/ihj.15-108

Krupp, M. D. (2005). Calcium influx and intracellular calcium transients. *Adv Exp Med Biol* 577, 157–164. doi: 10.1007/bf02982704

Lopez, J. R., Contreras, J., Linares, N., and Allen, P. D. (1999). Hypersensitivity of malignant hyperthermia-susceptible swine skeletal muscle to caffeine is mediated by high resting myoplasmic [Ca2+]. *Anesthesiology* 92, 1799–1806. doi: 10.1095/1999.42-20000600-00040

Mijares, A., Altamirano, F., Kolster, J., Adams, J. A., and Lopez, J. R. (2014). Age-dependent changes in diastolic Ca2+ and Na+ concentrations in dystrophic cardiomyopathy: Role of Ca2+ entry and IP3. *Biochem Biophys Res Commun* 452, 1054–1059. doi: 10.1016/j.bbrc.2014.09.045

Millay, D. P., Goonasekera, S. A., Sargent, M. A., Maillot, M., Aronov, B. J., and Molkentin, J. D. (2009). Calcium influx is sufficient to induce muscle dystrophy through a TRPC-dependent mechanism. *Proc Natl Acad Sci U S A* 106, 19023–19028. doi: 10.1073/pnas.0906591106

Mosole, S., Carraro, U., Kern, H., Loefler, S., Fruhmann, H., Vogelauer, M., et al. (2014). Long-term high-level exercise promotes muscle reinnervation with age. *J Neuropathol Exp Neurol* 73, 284–294. doi: 10.1097/nen.0000000000000032

Nicotera, P., and Orrenius, S. (1998). The role of calcium in apoptosis. *Cell Calcium* 23, 173–180. doi: 10.1016/s0143-4160(98)90116-6

Pierrangelo, L., D’incecco, A., Aimender, A., Michelacci, A., Kern, H., Dirksen, R. T., et al. (2015). Age-dependent uncoupling of mitochondria from Ca2+ entry units in skeletal muscle. *Onco-target* 6, 35358–35371. doi: 10.18632/oncotarget.6139

Rolland, Y., Czerwinski, S., Abellan Van Kan, G., Morley, J. E., Cesari, M., Onder, G., et al. (2008). Sarcopenia: its assessment, etiology, pathogenesis, consequences and future perspectives. *J Nutr Health Aging* 12, 433–450. doi: 10.1007/bf02982704

Ryan, M. J., Jackson, J. R., Hao, Y., Williamson, C. L., Dabkowski, E. R., Hollander, J. M., et al. (2010). Suppression of oxidative stress by resveratrol after isometric contractions in gastrocnemius muscles of aged mice. *J Gerontol A Biol Sci Med Sci* 65, 815–831. doi: 10.1093/gerona/glq086

Sauc, S., and Frieden, M. (2017). Neurological and Motor Disorders: TRPC in the Skeletal Muscle. *Adv Exp Med Biol* 993, 557–575. doi: 10.1007/978-3-319-57732-6_28

Shimamura, K., Zhou, M., Ito, Y., Kimura, S., Zou, L. B., Sekiguchi, F., et al. (2002). Effects of flufenamic acid on smooth muscle of the carotid artery isolated from spontaneously hypertensive rats. *J Smooth Muscle Res* 38, 39–50. doi: 10.1016/s0298-2704/02982704

Tutibbi, O., Brinkmeier, H., Rudel, R., and Fohr, K. J. (1999). Increased calcium entry into dystrophin-deficient muscle fibres of MDX and ADR-MDX mice is reduced by ion channel blockers. *J Muscle Res Cell Motil* 20, 389–398. doi: 10.1007/s10974-008-9076-0

Uryash, A., Flores, V., Adams, J. A., Pessah, I. N., and Lopez, J. R. (2020). Memory and Learning Deficits Are Associated With Ca2+ Dyshomeostasis in Normal Aging. *Front Aging Neurosci* 12:224.

Vanderbrouck, C., Martin, D., Colson-Van Schoor, M., Debaix, H., and Gaillly, P. (2002). Involvement of TRPC in the abnormal calcium influx observed in...
dystrophic (mdx) mouse skeletal muscle fibers. *J Cell Biol* 158, 1089–1096. doi: 10.1083/jcb.200203091

Visser, M., and Schaap, L. A. (2011). Consequences of sarcopenia. *Clin Geriatr Med* 27, 387–399. doi: 10.1016/j.cger.2011.03.006

Wanagat, J., Cao, Z., Pathare, P., and Aiken, J. M. (2001). Mitochondrial DNA deletion mutations colocalize with segmental electron transport system abnormalities, muscle fiber atrophy, fiber splitting, and oxidative damage in sarcopenia. *FASEB J* 15, 322–332. doi: 10.1096/fj.00-0320com

Wei, J., Xu, H., Davies, J. L., and Hemmings, G. P. (1992). Increase of plasma IL-6 concentration with age in healthy subjects. *Life Sci* 51, 1953–1956. doi: 10.1016/0024-3205(92)90112-3

Yang, T., Esteve, E., Pessah, I. N., Molinski, T. F., Allen, P. D., and Lopez, J. R. (2007). Elevated resting (Ca(2+))(i) in myotubes expressing malignant hyperthermia RyR1 cDNAs is partially restored by modulation of passive calcium leak from the SR. *Am J Physiol Cell Physiol* 292, C1591–C1598.

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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