Hyperammonemia Results in Reduced Muscle Function Independent of Muscle Mass

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Abstract

The mechanism of the nearly universal decreased muscle strength in cirrhosis is not known. We evaluated if hyperammonemia in cirrhosis causes contractile dysfunction independent of reduced skeletal muscle mass. Maximum grip strength and muscle fatigue response were determined in cirrhotic patients and controls. Blood and muscle ammonia concentrations and grip strength normalized to lean body mass were measured in the portacaval anastomosis (PCA) and sham operated, pair fed control rats (n=5 each). Ex-vivo contractile studies in the soleus muscle from a separate group of Sprague Dawley rats (n=7) were performed. Skeletal muscle force of contraction, rate of force development and rate of relaxation were measured. Muscles were also subject to a series of pulse trains at a range of stimulation frequencies from 20 to 110Hz.

Cirrhotic patients had lower maximum grip strength and greater muscle fatigue than control subjects. PCA rats had a 52.7±13% lower normalized grip strength compared to control rats and grip strength correlated with the blood and muscle ammonia concentrations (r²=0.82). In ex-vivo muscle preparations, following a single pulse the maximal force, rate of force development and rate of relaxation were 12.1±3.5g vs. 6.2±2.1g; 398.2±100.4g/s vs. 163.8±97.4g/s; -101.2±22.2g/s versus -33.6±22.3g/s in ammonia-treated compared with control muscle preparation respectively (p<0.001 for all comparisons). Tetanic force, rate of force development and rate of relaxation were depressed across a range of stimulation from 20-110 Hz. These data provide the first direct evidence that hyperammonemia impairs skeletal muscle strength and increased muscle fatigue and identifies a potential therapeutic target in cirrhotic patients.

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Introduction

The estimated prevalence of cirrhosis in the United States in 2010 was 0.27% which corresponds to 633,000 adults (33). Muscle strength in cirrhotics is not only reduced but is also an independent predictor of adverse clinical outcomes including decreased survival rates (2, 4, 10, 22). In addition, subjective fatigue is reported (23) but there are no published data on objective measures of skeletal muscle fatigability in cirrhosis. However, reduced muscle strength contributes to reduced exercise tolerance and activities of daily living which ultimately impairs quality of life (6, 21). Despite recognition of the high clinical significance of impaired skeletal muscle contractile function, there are no effective therapies because the specific mechanisms are poorly understood (3, 21, 28).

We have previously shown that hyperammonemia, due to hepatocellular dysfunction and portosystemic shunting in liver disease (27), is a mediator of the liver-muscle axis and is responsible for sarcopenia in cirrhosis with portosystemic shunting (10). Hyperammonemia activates skeletal muscle proteolysis by autophagy and upregulates myostatin expression that impairs protein synthesis (10, 31) with consequent sarcopenia. Thus, sarcopenia is, at least, partially responsible for the impaired grip strength observed in the hyperammonemic portacaval anastomosis (PCA) rat (10, 11), a model that allows us to dissect the consequences of portosystemic shunting from the necroinflammatory responses of cirrhosis (9). Skeletal muscle excitation/contractile dysfunction, in addition to sarcopenia, may also be a determinant of muscle strength, but there are no studies that have systematically examined contractile function in cirrhosis. Previous reports have, however, shown that ammonia
depolarizes the membrane potential resulting in reduced excitability of muscle fibers in response to electrical stimulation (18, 36) and reduced contractility of rat diaphragm muscle (35).

The purpose of this investigation was to determine if hyperammonemia in cirrhosis was a mediator of the impaired skeletal muscle contractile function independent of reduced muscle mass. We used a comprehensive array of models including human cirrhosis, the hyperammonemic portacaval anastomosis rat and ex-vivo muscle preparation. Specifically, we measured maximum grip strength and contractile strength after repetitive submaximal contraction in patients with cirrhosis and controls. We then examined if grip strength normalized to skeletal muscle mass (quantified as lean body mass) (12) is reduced in hyperammonemic PCA rats. Others have reported that ammonia impairs skeletal muscle contraction (18, 35). In the present study we quantified force production, rate of force development and rate of relaxation in ex-vivo skeletal muscle preparation. We hypothesized that maximum grip strength will be lower and muscle fatigue greater in human cirrhosis compared with controls. We also hypothesized that grip strength normalized to muscle mass will be lower in the PCA rat and inversely related to muscle and blood ammonia concentrations.
Methods

Human studies

Patients with cirrhosis as well as age and gender matched healthy controls were recruited for this investigation. Cirrhosis was diagnosed by liver biopsy and/or clinical, laboratory and imaging criteria. The clinical and demographic details of the subjects are shown in Table 1. Maximum grip strength was measured in the non-dominant hand using a Jamar® hydraulic hand dynamometer (Jamar Plus+; Sammons Preston, Rolyon, Bolingbrook, IL). After the initial maximum grip, subjects continued with hand grips at 40% of maximum grip every 2 seconds for 3 minutes, and the post-repetitive contraction final maximum grip strength was measured. The initial and final maximum grip strength and the difference between the two readings were quantified. All studies were approved by the Institutional Review Board of the Cleveland Clinic and a written informed consent was obtained in all subjects.

Animals

All studies were approved by the Institutional Animal Care and Use Committee at the Cleveland Clinic and the Cleveland Veterans Affairs Medical Center and conformed to animal care guidelines established by the National Institutes of Health. Male Sprague Dawley rats (~9 weeks old; 250-260g) with an end to side PCA or sham surgery (Charles River, Inc.; Wilmington, DE) were housed individually in a 12h light/dark cycle. The care of these animals has been described previously (9, 11). In brief, pair feeding was done by providing the sham operated control rats with the same quantity of standard rat chow (Harland Teklad rodent chow #8604; protein 24.5%, fat 4.4%, 3.93 Kcal/g) as had been ingested by a paired PCA rat fed ad libitum. Pair
feeding eliminated the effect of differences in food intake on measures of body composition and muscle function. Food and water intake were measured daily, and total body mass and lean body mass were measured weekly (Table 2). Lean body mass was obtained weekly using the TOBEC® body analyzer for small animals (SA-3000) fitted with the 114 x 318 mm measuring chamber (SA-3114) (EC Systems, Inc., Springfield IL).

Grip Strength

Grip strength was measured in the PCA and sham operated control rats at 4 weeks after the surgery using a computerized rat grip strength meter (model 1067CSX, Columbus Instruments, Columbus OH) as described previously (11). In brief, a supported T-bar was attached to the load cell with a sampling rate of 1000Hz. After animals were acclimatized to handling the T-bar with their front limbs the rat was then pulled away from the bar with gradually increasing force by holding the base of the tail until the rat released the bar. The maximal force, the force just prior to release of the T bar, was recorded. Each animal was tested three times in succession and then repeated twice after a 30 minute rest period. All measurements were performed by a single operator.

Euthanasia and harvesting

Following grip strength measurements, animals were euthanized, aortic blood samples were drawn into EDTA coated vials, plasma separated, aliquoted and frozen at -80°C, gastrocnemius muscle and other organs rapidly harvested, blotted dry of blood, weighed and flash frozen for further assays. A part of the muscle was collected in OCT® compound, frozen in isopentane chilled in liquid nitrogen. 10μm cryosections
were stained with ATPase at pH 10.5 (type II fibers stain dark, type 1 fibers stain light) and fiber size and typing done using Image J program (34). Ammonia concentrations were measured in plasma and muscle as described earlier (30). In brief, skeletal muscle protein content was quantified by the bicinchoninic acid method and muscle ammonia concentrations normalized to protein content. Plasma ammonia was quantified in deproteinized samples using a commercially available ammonia assay kit (Sigma Aldrich, St. Louis, MO). All assays were done in triplicate and the data expressed as mean±SD.

**Ex-vivo rat soleus contractile function**

A separate group of adult male Sprague Dawley rats (n = 8, age 12 weeks, weight 356 ± 35g) were matched in age and weight to the sham operated rats at the time of euthanasia (Table 2). Rats were well-anesthetized using a rodent anesthetic cocktail mixture (initial dose, ketamine 21–30 mg/kg, xylazine 4.3–6.0 mg/kg and acepromazine 0.7–1.0 mg/kg, with supplemental smaller doses given as needed to produce and maintain a deep level of anesthesia). The soleus muscles were removed from both legs with intact tendons and placed in oxygenated physiological solution and subsequently mounted vertically in separate double-jacketed tissue baths with one end attached to an isometric force transducer (Kent Scientific/Radnotti Glass Technology, Monrovia, CA). The composition (in mM) of the physiological solution was as follows: 135 NaCl, 5 KCl, 2.5 CaCl2, 1 MgSO4, 1 NaH2PO4, 15 NaHCO3, and 11 glucose, with the pH adjusted to 7.35-7.45 at 37°C while being aerated with 95% O2-5%CO2. Temperature of the medium was maintained at 37°C throughout the study period. Muscle was stimulated by placement of platinum electrodes (Radnotti Glass
Technology, Monrovia, CA) directly on the muscle parallel to the longitudinal axis. Optimal length for each muscle, defined as the muscle length associated with maximum twitch force, was determined by delivering series of single supramaximal voltages with a pulse width of 1 ms. Muscles were lengthened between subsequent stimulations until the twitch force was maximized. The muscles then remained at optimal length for the duration of the contractile study.

Following equilibration, twitches were evoked every minute for a period of 5 minutes to determine baseline force and confirm contractile stability of the muscle. The right sided muscle served as the test group and the left sided muscle from the same animal served as a control. After this 5 minute baseline period, ammonium acetate in Kreb’s buffer solution was added to the bath of the right sided muscle to a final concentration of 10 mM. An equal volume of Kreb’s solution alone was added to the bath of the left sided control muscle. We have previously shown in vitro in C2C12 myotubes that 10mM ammonium acetate in the medium results in intracellular ammonia concentrations that are similar to that in the skeletal muscle of patients with cirrhosis (30). Consistently, incubation of skeletal muscle in Kreb’s solution with 10mM ammonium acetate resulted in muscle ammonia concentration of 5.01±0.65 mM/g protein which is similar to that in human cirrhotic skeletal muscle of 4.98±0.41 mM/g protein (30). Following addition of ammonium acetate or control solution, twitch stimulation continued once every minute for 20 minutes.

Following this initial twitch protocol the muscles were then subject to a series of tetanic contractions by stimulating the muscle for 300ms at stimulation frequencies of 20, 35, 50, 65, 80, 95, and 110 Hz to develop a force frequency relation. A one minute
rest period was provided between each contraction. Following all contractile protocols the length and mass of each muscle were recorded after this study and the ammonia concentration in the muscle tissue quantified as described earlier (30).

Force records were collected online with a Powerlab 8/35 data acquisition system and analyzed using the associated Labchart® software (AdInstruments, Colorado Springs, CO). For all twitch and tetanic contractions, maximal force, rate of force development and rate of relaxation were determined. Specifically the rate of force development was calculated as the rate in which force increased from 10% to 90% of maximal force. Likewise, the rate of relaxation was the rate in which force decreased from 90% to 10% of maximal force following stimulation. In addition, twitch force following exposure to hyperammonemia was normalized to the average twitch force during the 5 minute baseline period. Force-frequency relationships with and without the drug were determined by measuring peak force across all stimulation frequencies (20-110Hz). Since muscle mass and initial force generated on the two sides from the same animal were similar and each treated muscle was matched to the control muscle from the contralateral limb of the same animal, force was not normalized to muscle mass or cross sectional area.

Immunoprecipitation for nitration of myosin heavy chain.

To determine if the major contractile protein, myosin heavy chain (38), is post-translationally modified by ammonia, total protein was extracted from C2C12 murine myotubes grown to differentiation for 48 h as previously described (30). Cells were then treated with 10mM ammonium acetate for 24 h. We have previously reported that this concentration of ammonium acetate results in a cellular ammonia level similar to that in
the skeletal muscle of human cirrhotics (30) and in the PCA rat (present study). For immunoprecipitation studies, 20 µl of protein A/G agarose beads were added to 500µl of protein lysate (200µg protein), and incubated in a shaker for 1 hour at 4°C. This was followed by centrifugation at 14,000 g at 4°C for 5 minutes. The bead pellet was discarded and to the supernatant 2 µg affinity-purified polyclonal antmyosin heavy chain antibody (Cat No: 05-716, Millipore, Billerica, MA) (1:200) and incubated for 1 hr at 4°C by rotating. Protein A/G agarose beads were then added and the lysate-beads mixture was incubated at 4°C under rotary agitation overnight. The lysate bead mixture was then centrifuged, supernatant removed and beads washed 3x in lysis buffer. About 50 µl of 2x loading buffer was added to the washed bead, boiled for 5 minutes to denature the protein and separate it from the protein-A/G beads. Following centrifugation, the supernatant containing the immunoprecipitated myosin heavy chain was run on an 8% tris glycine gel, electrotransferred to PVDF membrane, incubated with anti nitrotyrosine antibody (Cat no: 05-233, Millipore, Billerica, MA) (1:10,000) at 4°C overnight and then treated with secondary antibody and developed using enhanced chemiluminescence plus solution and blots developed. The membrane was also probed for myosin heavy chain to ensure equal loading of protein and non-specific immunoglobulin was used as a negative control.

Data analysis

All data were expressed as mean±SD unless specified. Chi square test was used to compare qualitative and the Student’s ‘t’ test for independent groups was used to compare quantitative variables. Normalized grip strength was correlated with blood and skeletal muscle ammonia concentrations across both control and PCA rats using the
Pearson’s correlation coefficient. To determine if hyperammonemia causes muscle
dysfunction, twitch force, rate of force development and rate of relaxation were
compared between control and ammonia acetate treated muscles with mixed-design
ANOVAs followed by post hoc comparisons between groups. Benjamini-Hochberg α
correction was used to minimize the chance of a type 1 error. In regards to the force-
frequency relationship, two-way mixed design ANOVAs were performed to determine if
ammonia acetate influenced muscular force, rate of force development and rate of
relaxation across a range of stimulation frequencies. The alpha level for all comparisons
was set at α=0.05.

Results

Human Studies.

Contractile strength measurements are shown in table 1. Initial maximum grip
strength and post repetitive contraction grip strength were both significantly lower
(p<0.001) in patients with cirrhosis than in controls. Additionally, 4 out of 16 (25%) patients with cirrhosis could not complete the repetitive contraction component due to
extreme muscle fatigue in the hand while all control subjects completed the study. All 4
subjects who could not complete the study had Child’s score ≥7. The difference
between the initial maximum and post repetitive contraction grip strength (expressed as
a percentage of the initial grip strength) was also significantly greater in patients with
cirrhosis than controls. Since the number of subjects was small, it was not possible to
determine if contractile dysfunction was related to the etiology of cirrhosis.

PCA rat grip strength and ammonia concentrations

Plasma and muscle ammonia concentrations in the PCA were significantly higher
than in sham operated control rats (Table 2). There was a strong correlation between plasma and skeletal muscle ammonia concentrations ($r^2=0.88; p<0.01$ in the PCA, $r^2=0.64; p<0.05$ sham; and $r^2=0.89; p<0.01$ when both groups were combined). Grip strength normalized to lean body mass was significantly reduced in the PCA rats (2.22±0.62 grams/gram lean body mass) compared to control rats (4.54±0.99 grams/gram lean body mass, $P=0.001$) (Figure 1 A). Furthermore, there was a strong inverse correlation ($r^2=0.81, P=0.002$) between blood ammonia concentration and grip strength when the PCA and control rats were pooled together (Figure 1 B). Similarly, muscle ammonia concentrations were inversely correlated with grip strength ($r^2=0.82; p<0.01$). Cryosections of gastrocnemius muscle from the PCA rat showed a reduction in fiber diameter compared to sham operated control rats. There was also loss of type II fibers with no inflammatory cell infiltration or myonecrosis (Figure 2 A, B)

**Ammonium acetate treated isolated muscle**

The mass of the soleus muscles were not different between control (0.124±0.033g) and ammonium acetate treated (0.121±0.037g) conditions ($p=0.67$). Furthermore the twitch force during the initial 5 minutes baseline period was also not different between the two groups (control 15.5±6.4g, hyperammonemia: 15.3±6.2g, $p=0.906$). Post analysis of the muscle tissue revealed that the ammonia concentration in control muscles was 0.39±0.08 mM/g protein and 4.7±0.7 mM/g protein in the ammonia treated muscles ($p<0.001$).

Within 8 minutes of exposure to hyperammonemia, muscles generated significantly less twitch force than their control counterparts ($p=0.02$). By the end of the 20 minute protocol, muscles treated with ammonium acetate were producing 41.5±7.6%
of baseline force whereas the control pairs were producing 84.2±24.2% of baseline (p<0.001) (Figure 3 A,B). The reduction in muscle twitch force stabilized by 20 minutes. In addition, within 11 minutes the muscles treated with ammonium acetate had a reduced rate of force development (p=0.044) and rate of relaxation (p=0.037) compared to control muscles (Figure 4 A, B). During the subsequent 9 minutes the rate of force development and rate of relaxation continued to decrease and at 20 minutes exposure to hyperammonemia, the rates were 40±24% and 34±23% of that in the control muscles, respectively.

**Force-Frequency Relationship**

The reduction in force, rate of force development and rate of relaxation observed with the single twitch contractions was also observed when the muscles were stimulated with pulse trains across a range of stimulation frequencies (20-110Hz). Specifically, across all stimulation frequencies, the tetanic force was reduced between 55% and 64% in the muscles treated with ammonium acetate compared to controls (p<0.008 for all comparisons) (Figure 5 A). In addition, the rate of force development was depressed between 56% and 60% and the rate of relaxation depressed between 69% and 71% across all stimulation frequencies in the muscles treated with ammonia acetate (p≤0.009 for all comparisons; Figure 5 B,C).

**Nitration of myosin heavy chain.**

Immunoprecipitation studies showed nitration of myosin heavy chain during hyperammonemia compared to controls cells suggesting that post translational modifications of proteins may contribute to impaired contractile function (Figure 6 A,B).
Discussion

The present studies show that patients with cirrhosis have lower grip strength and importantly, more rapid muscle fatigue compared to healthy controls. The hyperammonemic PCA rats have lower grip strength when normalized to lean body mass when compared to control rats and grip strength correlated inversely with the blood and skeletal muscle ammonia concentrations. Consistently, our *ex-vivo* experiments revealed that skeletal muscle force production, rate of force development and rate of relaxation following single pulses and pulse trains across a range of stimulation frequencies was lower during hyperammonemia. Finally, hyperammonemia resulted in nitration of myosin heavy chain, a major contractile protein in the skeletal muscle. Together, these data indicate that hyperammonemia is a mediator of reduced muscle contractile function independent of reduced muscle mass and provides a potential therapeutic target to reverse muscle weakness in cirrhosis.

Our human data, which indicates patients with cirrhosis have significantly lower grip strength than controls, was consistent with previous reports (2, 4). This is however, the first study that evaluated skeletal muscle response to repetitive contraction and fatigue. Nearly a third of patients with cirrhosis could not perform the 3 min repetitive submaximal contraction while all the controls were able to complete the study. Interestingly, the maximum grip strength following 3 minutes of submaximum repetitive contractions was reduced in both groups, the reduction was significantly greater in cirrhotic patients. One of the mechanisms of muscle fatigue in response to contraction has been suggested to be due to increased muscle ammoniagenesis. Since we have previously shown that muscle ammonia concentration in the skeletal muscle of cirrhotics...
is significantly higher than in controls (30), our current PCA rat and ex-vivo studies indicate that higher muscle hyperammonemia was a mechanism of the reduced strength and increased fatigue observed in those with cirrhosis.

Models used.

We have used 2 approaches to identify that ammonia is responsible for specific contractile dysfunction associated with liver disease: in-vivo studies in the PCA rat and ex-vivo studies in isolated skeletal muscle. Our in-vivo PCA rat is an established model of hyperammonemia of portosystemic shunting in liver disease that reproduces the clinical, biochemical, hormonal and metabolic perturbations of cirrhosis that are due to the vascular consequences of cirrhosis. Even though cirrhosis does not develop in the PCA rat, the major strength of this model is that it allows us to dissect the vascular consequences from the necroinflammatory effects of cirrhosis. Rodent models of cirrhosis include the carbon tetrachloride induced fibrosis, which is not an appropriate model because carbon tetrachloride is directly toxic to the skeletal muscle (32), and the bile duct ligation which has steatorrhea that makes it difficult to separate the muscle atrophy due to malabsorption from that secondary to cirrhosis (15). Furthermore, since the perturbations in the PCA rat are established at surgery (11, 26), a time course can be defined and based on our previous studies characterizing this model (9, 11-14), the variability within the disease group is much less than would be when there is ongoing toxin administration or continued but variable steatorrhea. We have previously reported that the grip strength and skeletal muscle mass are lower in the PCA rat (11). The present studies show that lower grip strength in the PCA compared to sham operated control rats is independent of the lower muscle mass. Finally, our studies in patients
with cirrhosis allow direct translation of our animal and *ex-vivo* studies. Our novel data showed that grip strength normalized to lean body mass is decreased in the hyperammonemic PCA compared to that in control rats. This is also the first report of *in-vivo* studies that show grip strength is highly correlated with plasma and skeletal muscle ammonia concentrations. Our *ex-vivo* preparation was optimized so that muscle concentrations of ammonia were similar to that seen in patients with cirrhosis (30) and the PCA rat, which allowed us to examine the characteristics of skeletal muscle contractile dysfunction independent of reduced muscle mass. Our data show that maximal force, rate of force development and rate of relaxation are all reduced due to the influx of ammonia into the muscle. This complementary approach has thus allowed us to identify hyperammonemia as the specific mediator of contractile dysfunction in cirrhosis and potentially other hyperammonemic disorders.

*Skeletal muscle uptake and effects of ammonia in liver disease*

In patients with acute and chronic liver disease, the skeletal muscle contributes to ammonia disposal by increased uptake and metabolism to glutamate and glutamine that are then exported out of the muscle (7, 16, 19, 24). We and others have reported skeletal muscle hyperammonemia impairs skeletal muscle protein synthesis and increases autophagy that contributes to sarcopenia in cirrhosis (20, 30, 31). Consistent with previous data on increased uptake of ammonia by the skeletal muscle, muscle ammonia concentrations in the PCA rat was significantly higher than in sham operated controls. Our data also show that lower skeletal muscle mass in the PCA rat was accompanied by reduced fiber diameter, but there was no evidence of myonecrosis or inflammatory infiltrate indicating that the effects are due to loss of protein content rather
than a direct injury to the muscle. Furthermore, the loss of type IIB fibers is similar to that reported in other models of cirrhosis (17). The present studies provide compelling novel data that, hyperammonemia alters skeletal muscle contractile function and increases fatigue independent of the loss of muscle mass. Our data are also important because, even though ammonia has been previously suggested to cause fatigue following exercise (5, 37), it is not known if this is responsible for the muscle dysfunction in patients with cirrhosis. Cerebral disposal of ammonia maybe adequate during limited ammonia generation during intense exercise (8) but in hepatic disease, skeletal muscle becomes a major alternate organ for ammonia disposal (7). Our ex vivo studies show that increased muscle uptake of ammonia directly impairs contractile response, the major non-metabolic skeletal muscle mechanical function.

Contractile dysfunction during hyperammonemia

Previous reports have suggested that physiological concentrations of ammonia do not adversely affect diaphragm contractility (35). Our data are however consistent with this report that ammonia impairs in vitro diaphragm contractile function only at concentrations above 5mM in the medium which are not in the physiological range of plasma. A true measure of the effects of ammonia is however the muscle tissue concentration. In cirrhosis, muscle uptake and concentrations are significantly higher than that following exercise and our studies using the biologically appropriate muscle concentrations provide direct evidence that contractile dysfunction \textit{ex-vivo} and lower \textit{in-vivo} grip strength in portosystemic shunting in liver disease are mediated by hyperammonemia. This interpretation is consistent with a previous report of decrease in diaphragm contractility following exposure to 5, 10 and 14mM ammonium chloride when
both the rate of force development and rate of relaxation were impaired following exposure to ammonium acetate (35). To demonstrate that ammonia results in impaired contractile function independent of loss of muscle mass, grip strength was normalized to lean body mass and gastrocnemius muscle mass. We have previously shown that lean body mass was a non-invasive measure of skeletal muscle mass in rats (12) and by normalizing the muscle strength to lean body mass, we noted that hyperammonemia resulted in lower muscle contraction independent of muscle loss \textit{in-vivo} in the PCA rat.

\textbf{Electrophysiology of ammonia induced muscle dysfunction}

A potential mechanism for the hyperammonemia induced decrease in muscle contractility is a reduction in membrane potential (1, 18). Heald (1975) reported that ammonium ions can either potentiate or depress contractile function depending on their concentration. Specifically frog sartorius muscle fibers were potentiated when 30mM NH$_4$Cl replaced NaCl in the bathing solution but was depressed when much higher concentrations of ammonium chloride (90 and 120 mM) replaced NaCl. The reduction in force at higher concentrations was due to depolarization of the resting membrane potential and subsequent loss of excitable fibers. In addition, based on the fact that outward membrane currents could produce contractions and that hyperammonemia did not block caffeine induced contractures, Heald concluded that the surface membrane, not the sarcoplasmic reticulum or contractile apparatus is the major site of interference by ammonium. It is important to note that the concentrations of ammonium chloride used by Heald were much greater than what has been reported in human cirrhosis (30). However, Stephenson and Stephenson (1996) reported that the magnitude of twitch force did not differ between control fibers and fibers exposed to 2-10 mM NH$_4$OH, yet
the twitch duration was reduced by 11-25%. They concluded that changes in the twitch
duration may reflect changes in voltage sensor activation, changes in the SR induced
calcium movements or changes to calcium sensitivity of actin. Two major differences in
the muscle preparation between our investigation and that of Heald’s and Stephenson’s
is our use of whole muscle, not muscle fibers, and we simply added ammonium acetate
to the bath solution rather than replace sodium with the ammonium ion.

**Potential mechanisms of ammonia induced contractile dysfunction**

Muscle contraction is an energy intense process and impaired mitochondrial
function and ATP content have been reported in the skeletal muscle of patients with
cirrhosis. Ammonia also can potentially cause post-translational modifications including
protein nitration and oxidative stress induced carbonylation of contractile proteins with
impaired actomyosin interactions. We noted nitration of major structural protein, myosin
heavy chain in myotubes in response to hyperammonemia. Since nitration of proteins
has been reported to impair function, ammonia mediated nitration is a potential
molecular mechanism of impaired contractile function (25). Future studies will allow
dissection of the specific molecular and biochemical mechanisms of ammonia induced
impaired mechanical function of the muscle.

**Strengths and limitations of the study**

The major strength of these studies is the identification of ammonia as a
mediator of abnormal muscle contractile function in cirrhosis with portosystemic
shunting that provides the rationale for using ammonia lowering therapies to improve
muscle strength. This is reiterated by a previous report suggesting that following liver
transplantation, when hepatic ammonia metabolism normalizes, grip strength
normalizes even before muscle mass improves (29). Since ammonia lowering therapies are routinely used in treating patients with cirrhosis and encephalopathy, our studies have the potential for rapid clinical translation. A potential limitation is that the exact molecular mechanism by which ammonia impairs contractile function and accelerates fatigue is not known. However, our novel data lay the foundation for studies to determine the mechanisms(s) of ammonia induced impaired muscle contractile and fatigue responses in cirrhosis and potentially other chronic diseases with hyperammonemia. Even though cirrhosis is a complex disease with multiple metabolic perturbations, ammonia is the best established mediator to date of the liver-muscle axis. Other mechanisms may also contribute to impaired contractile function in liver disease but our studies show that hyperammonemia does contribute to contractile dysfunction.

Key findings of the study

These data provide evidence for ammonia as a mediator at least partially responsible for the impaired contractile function of the skeletal muscle in cirrhotics. A number of potential mechanisms can contribute to the impaired muscle contraction and relaxation, and include ammonia induced impairment in mitochondrial function and ATP generation, post translational modifications of critical contractile proteins and altered membrane potential. Our studies lay the foundation for mechanistic studies to reverse impaired muscle contraction and relaxation during hyperammonemia. These data are of broad interest because hyperammonemia has been reported in other chronic disorders including advance heart failure and chronic obstructive lung disease, both of which have sarcopenia and impaired contractile function.
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Figure Captions

Figure 1: Grip strength for PCA and sham treated rats. A) Grip strength (mean±sd) normalized to lean body mass (n=6). * indicate a significant difference between the two conditions (P=0.001). B) Correlation between normalized grip strength and blood ammonia concentration across both control (triangles) and PCA (diamond) rats.

Figure 2: Immunohistochemistry of cryosections of gastrocnemius muscle of sham operated control and hyperammonemic, portacaval anastomosis (PCA) rat. A) Gastrocnemius muscle stained with ATPase at pH10.4. Dark stained type II fibers and light stained type I fibers showed loss of type II fibers and reduction in diameter of both fiber types. B) Quantification of fiber diameter in gastrocnemius muscle of the PCA rats expressed as a percentage of that from sham operated control rats. Data from at least 120 fibers (mean±SD). *** p<0.001.

Figure 3: Influence of ammonium acetate on twitch force. Time -5 through 0 represent the 5 minute baseline period. At time 0 either ammonia acetate or kreb solution was added to the muscle bath. (A) Illustrates the absolute twitch force. (B) Illustrates the relative change from baseline for the two conditions. * indicate significant difference in force between the muscle treated with ammonia acetate and those treated with normal Kreb solution. Values are presented as mean±sd.

Figure 4: Influence of ammonium acetate on rate of force development (A) and rate of relaxation (B). Time -5 through 0 represent the 5 minute baseline period. At time 0 either ammonia acetate or kreb solution was added to the muscle bath. * indicate significant difference in force between the muscle treated with ammonia acetate and those treated with normal Kreb solution. Values are presented as mean±sd.

Figure 5: Influence of ammonium acetate on tetanic force (A), rate of force development (B) and rate of relaxation (C) across a range of stimulation frequencies. Across all stimulation frequencies statistical significance was achieved for all comparisons between muscles treated with Kreb solution and those treated with ammonia acetate. Values represent mean±sd.

Figure 6: Representative immunoblots and densitometry of nitrated myosin heavy chain (MyHC). A) Myosin heavy chain was immunoprecipitated from C2C12 myotubes treated with 10mM ammonium acetate and control cells for different time points (0-24h). Immunoprecipitates were then probed with antinitrotyrosine antibody. IgG was used as a negative control and myosin heavy chain was probed to demonstrate equal loading. B) Densitometry from 3 independent experiments showing nitration of myosin heavy chain in ammonia treated myotubes.
## Table 1. Clinical and demographic characteristics of patients with cirrhosis and control subjects

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<td>NASH 5</td>
<td>Alcohol 7</td>
</tr>
<tr>
<td></td>
<td>Viral hepatitis 2</td>
<td>Primary biliary cirrhosis 2</td>
</tr>
<tr>
<td>Child-Pugh score</td>
<td>8.3±1.5</td>
<td></td>
</tr>
<tr>
<td>MELD score</td>
<td>8.8±1.9</td>
<td></td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>29.4±3.9</td>
<td>27.6±3.6***</td>
</tr>
<tr>
<td>Initial grip strength (kg)</td>
<td>35.6±11.4</td>
<td>21.6±9.2***</td>
</tr>
<tr>
<td>Final grip strength (kg)</td>
<td>31.2±9.2</td>
<td>17.7±9.5***</td>
</tr>
<tr>
<td>Unable to complete study</td>
<td>0</td>
<td>4 (25%)***</td>
</tr>
<tr>
<td>Final grip strength (% initial)</td>
<td>89.8±10.3</td>
<td>77.7±19.5</td>
</tr>
</tbody>
</table>

All values are mean±SD  *** p<0.001
Table 2. Characteristics of PCA and sham rats.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Sham</th>
<th>PCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Initial body weight (g)</td>
<td>256±5.3</td>
<td>254.6±5.1</td>
</tr>
<tr>
<td>Initial lean body mass (g)</td>
<td>222.2±7.0</td>
<td>219.2±6.1</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>387.0±28.3</td>
<td>282.4±21.1***</td>
</tr>
<tr>
<td>Final lean body mass (g)</td>
<td>323.6±26.1</td>
<td>256.2±23.11***</td>
</tr>
<tr>
<td>Total food intake (g)</td>
<td>567.4±18.1</td>
<td>575.1±18.3</td>
</tr>
<tr>
<td>Average daily food intake (g)</td>
<td>20.4±0.7</td>
<td>20.3±0.7</td>
</tr>
<tr>
<td>Food efficiency</td>
<td>0.23±0.05</td>
<td>0.046±0.045***</td>
</tr>
<tr>
<td>Total water intake (ml)</td>
<td>725.6±10.1</td>
<td>722.8±8.2</td>
</tr>
<tr>
<td>Daily water intake (ml)</td>
<td>25.8±0.29</td>
<td>25.9±0.36</td>
</tr>
</tbody>
</table>

All values mean±SD  *** p<0.001.
A  

**Force (g)**  

0 5 10 15 20 25  

*************  

**B**  

**Relative Force (%)**  

0 20 40 60 80 100 120  

*************  

- **Ammonium acetate**  
- **Control**  

**Time (s)**  

-5 0 5 10 15 20
A  

**Df/Dt 10-90% (g/s)**

- Ammonium acetate
- Control

B  

**Rate of relaxation 90-10% (g/s)**