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Ammonia lowering reverses sarcopenia of cirrhosis by restoring skeletal muscle proteostasis

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Abstract

Sarcopenia or skeletal muscle loss is a frequent, potentially reversible complication in cirrhosis that adversely affects clinical outcomes. Hyperammonemia is a consistent abnormality in cirrhosis that results in impaired skeletal muscle protein synthesis and breakdown (proteostasis). Despite availability of effective ammonia lowering therapies, whether lowering ammonia restores proteostasis and reverses muscle mass is unknown. Myotube diameter, protein synthesis and molecular responses in C2C12 murine myotubes to withdrawal of ammonium acetate following 24 h exposure to 10mM ammonium acetate were complemented by *in vivo* studies in the hyperammonemic portacaval anastomosis rat (PCA) and sham operated, pair-fed (SO) Sprague-Dawley rats treated with ammonia lowering therapy by L-ornithine L-aspartate and rifaximin orally for 4 weeks. We observed reduced myotube diameter, impaired protein synthesis and increased autophagy flux in response to hyperammonemia that were partially reversed following 24h and 48h withdrawal of ammonium acetate. Consistently, 4 weeks of ammonia lowering therapy resulted in significant lowering of blood and skeletal muscle ammonia, increase in lean body mass, improved grip strength and higher skeletal muscle mass, diameter and an increase in type II fibers in the treated compared to untreated PCA rats. Increased skeletal muscle myostatin expression, reduced mTORC1 function, and the hyperammonemic stress response including autophagy markers were also reversed in the PCA rats treated with ammonia lowering therapy. Despite significant improvement, molecular and functional readouts were not completely reversed by ammonia lowering measures.

Conclusions—Ammonia lowering therapy results in improvement in skeletal muscle phenotype, function and molecular perturbations of hyperammonemia. These preclinical studies complement previous studies on ammonia induced skeletal muscle loss and lay the foundation for prolonged ammonia lowering therapy to reverse sarcopenia of cirrhosis.

Sarcopenia or loss of skeletal muscle mass is a frequent complication of cirrhosis, advanced heart failure and chronic lung disease that adversely impacts outcomes^(1–12). Despite the high clinical significance, there is limited data on our understanding of the mechanisms of sarcopenia and consequent lack of effective therapies⁽³⁾. Hyperammonemia is a consistent abnormality in cirrhosis due to impaired ureagenesis capacity and portosystemic shunting^(13–15). Skeletal muscle mass is maintained by proteostasis, a cellular homeostatic process that includes protein synthesis, folding, trafficking and breakdown. We and others have previously reported that hyperammonemia impairs skeletal muscle proteostasis by decreased protein synthesis and increased autophagy with consequent sarcopenia^(16–19). Skeletal muscle hyperammonemia results in transcriptional upregulation of myostatin. Increased expression of myostatin, a TGF β superfamily member, contributes to impaired mTORC1 signaling and consequent reduction in muscle protein synthesis^(20, 21). Additionally, phosphorylation of eIF2 α during hyperammonemia contributes to impaired protein synthesis and sarcopenia^(17, 22, 23). Our previous studies have shown that during hyperammonemia, both impaired mTORC1 signaling and increased eIF2 α phosphorylation impair protein synthesis^(17, 24).

Even though hyperammonemia impairs proteostasis, it is not known if lowering ammonia will reverse skeletal muscle molecular and metabolic perturbations and consequent sarcopenia. Ammonia lowering strategies have been used in preclinical and clinical studies to treat hepatic encephalopathy, a recognized consequence of hyperammonemia⁽²⁵⁾. However, there are no reports of skeletal muscle responses to ammonia lowering therapy. Of the ammonia lowering agents used in clinical practice, lactulose therapy is difficult to monitor in animals and hence a combination rifaximin by gavage and L-ornithine L-aspartate (LOLA) in the drinking water was used. Rifaximin and LOLA have been reported in rats to lower ammonia and reverse the cerebral effects of hyperammonemia^(26, 27). Despite compelling evidence that hyperammonemia induces skeletal muscle metabolic and molecular perturbations^(16, 17, 19), it is not known if lowering ammonia will reverse these responses and consequent sarcopenia. Current options to reverse sarcopenia have focused on nutritional and amino acid supplementation with limited benefits⁽²⁸⁾.

There are no published studies on the effects of ammonia lowering therapies on body composition or skeletal muscle responses. In brain tissue, ammonia accumulates in cirrhosis and backflux (reversed transfer) from the brain into circulation has been reported⁽²⁹⁾ but whether ammonia lowering treatment results in reduction in muscle tissue concentrations and consequent reversal in molecular and functional perturbations are not known. In liver disease, muscle uptake of ammonia is increased^(30, 31) with molecular and metabolic perturbations^(16, 19). However, after liver transplantation that reverses the metabolic and hormonal perturbations of cirrhosis, sarcopenia does not reverse and may actually worsen⁽⁴⁾. These observations suggest that the effects of ammonia on the skeletal muscle may be irreversible even though other factors including the use of mTORC1 and calcineurin inhibitors after transplantation may replace hyperammonemia as the mediator of sarcopenia after transplantation⁽³⁾. The present studies were performed initially *in vitro* in murine myotubes exposed to hyperammonemia followed by withdrawal of ammonia *in vivo* to specifically determine if the responses were indeed reversible. Studies were then performed

in the portacaval anastomosis (PCA) rat, a model of sarcopenia in hyperammonemia of portosystemic shunting^(20, 32, 33).

Materials and methods

In vitro responses of myotubes to ammonia withdrawal

Murine C2C12 myoblasts (ATCC, Manassas, VA) were grown to confluence in proliferation medium (DMEM with 10% fetal bovine serum) followed by 48 h differentiation in DMEM with 2% horse serum⁽³⁴⁾. Differentiated myotubes exposed to 10mM ammonium acetate for 24 hours were used as reported previously^(16, 19). For the withdrawal experiments, myotubes treated with ammonium acetate for 24 h followed by removing and replacement with fresh differentiation medium for specified times. Myotube diameter was measured as described by us⁽¹⁶⁾ from at least 100 myotubes from 4 independent replicate plates and data expressed as percentage of untreated controls. Protein synthesis was quantified using a puromycin incorporation assay⁽¹⁷⁾. For signaling responses, cells were lysed, protein quantified by the bicinchoninic acid assay, and immunoblots for myostatin, total and phosphorylated eIF2 α and eIF2 α kinase (GCN2), AMPK, mTORC1 and its targets, p70S6 kinase, ribosomal S6 protein, expression of autophagy markers, LC3 lipidation and p62 as described earlier⁽¹⁷⁾. Autophagy flux was determined using lysosomal inhibitor, chloroquin at 25 μ M as previously described⁽³⁵⁾.

In vivo response to ammonia lowering therapy

Post-pubertal male Sprague-Dawley rats aged 9 weeks, with either end-to-side PCA or sham surgery were obtained from Charles River Inc. (Wilmington, MA). Animals were housed individually in a 12 h light/dark cycle as described earlier⁽³²⁾. Both groups of animals were fed standard rat chow (Harland Teklad rodent chow #8604; 24.5% protein, 4.4% fat, 3.93 Kcal.g⁻¹) with the PCA rats being fed ad libitum while the sham operated rats were pair fed (SO) to the PCA rats as described earlier⁽²⁰⁾. Food and water intake were measured daily. Body weight, body composition measured by total body electrical conductivity (TOBEC) and grip strength was measured weekly as described earlier^(20, 33) till euthanasia on the study day. Rifaximin (50mg.kg⁻¹.d⁻¹) in 1 ml of drinking water was administered by gavage daily for 28 days. Additionally, L-ornithine L-aspartate was added to the drinking water and the concentration was adjusted to deliver an amount of 2 g.kg⁻¹. d⁻¹. This dose and route of rifaximin and LOLA have been reported to lower blood ammonia in rats^(26, 27). The combination treatment with rifaximin and LOLA (r-LOLA) was started at 48 h after surgery. At the end of 28 days, on the night before the study day, food but not water was withdrawn to ensure uniform duration of postabsorptive phase in all animals. Animals were consistently euthanized between 10 AM and 11 AM to avoid circadian alterations in proteostasis in 8 animals in each group.

Protein synthesis *in vivo*—A flooding dose of sterile L-[ring-D₅] phenylalanine (300mM.kg⁻¹) was injected intraperitoneally and 30 min later, rats were euthanized by intraperitoneal pentobarbitone injection. Enrichment of D₅Phe was quantified in the cytosol and bound to protein by liquid chromatography mass spectrometry/mass spectrometry as previously described⁽²⁴⁾. In brief, muscle tissue was homogenized and deproteinized in 250

μl 0.098M trichloroacetic acid, containing 0.1 gram glass beads using a mini-Bead Beater (Biospec Products, Bartlesville, OK, USA) for 30 seconds and then centrifuged at 8000g at 4°C for 5 min. The protein pellet was then washed 3 times with 0.3 M TCA and proteins were hydrolyzed with 1 ml 6N hydrochloride solution at 100°C for 24 hours. After adding 5 ml HPLC grade water, samples were dried down with a vacuum concentrator (SpeedVac, Thermoscientific, Waltham, MA, USA). Samples were then dissolved in 20μl water per mg wet tissue weight, centrifuged at 50,000g 4°C for 5 min. and 20μl of the supernatant was used for the tracer/Tracee (*t/T*) analyses of protein-bound phenylalanine by liquid chromatography/mass spectrometry/mass spectrometry.

Samples and enriched standards with *t/T* ratios of L-[ring-²H₅]-phenylalanine (D₅Phe) in expected ranges (calibration curve for *t/T* measurements), were derivatized using 9-fluorenylmethoxycarbonyl (Fmoc). Electrospray triple quadrupole tandem mass spectrometry in multiple reactions monitoring mode was used for detections. Area under the curve of a mass signal was used to calculate *t/T* using the D₅Phe calibration curve. Quality control of the Fmoc derivatization procedure was done using the mass signal of the L-[D₈]-valine.

Fractional synthesis *rate* (FSR) of protein synthesis was calculated by the formula:

$$\text{MPE}_{\text{bound}} / (\text{MPE}_{\text{free}} \times t)$$

where MPE_{bound} is the mole percent excess of D₅Phe in the muscle protein, MPE_{free} is the enrichment in the cytosol, and *t* is the exact time in hr. and expressed as %/h. Absolute synthesis rate (ASR) was calculated as the product of the FSR and the total muscle protein content as described earlier⁽²⁰⁾ and expressed as mg/h. The total protein in the skeletal muscle was measured using the BioRad DC assay (BioRad, Hercules, CA). Food efficiency (weight.gm food intake⁻¹) was calculated as previously described⁽²⁰⁾.

Protein synthesis *in vitro*—Puromycin incorporation was used to quantify protein synthesis in C2C12 myotubes by incubating the cells following treatment with ammonium acetate or medium alone, 1μM puromycin added in the final 30 min and immunoblots for puromycin incorporation performed as described earlier⁽¹⁷⁾.

Quantitative analyses

Ammonia concentrations were quantified using methods standardized in our laboratory⁽¹⁶⁾. Plasma free amino acids were quantified using ion-exchange chromatography (L-8900 Hitachi, Tokyo, Japan) with a post-column ninhydrin reaction (Wako, USA)⁽²⁴⁾.

Cryosections of gastrocnemius muscle were stained and minimal internal diameter, area on hematoxylin eosin stain, ratio of type II/type I fibers on ATPase stain were quantified as previously described⁽³⁶⁾. The percentage of fibrosis/muscle fiber areas was also determined.

Signaling responses—mTORC1 signaling was determined by phosphorylation of targets: phospho p70s6k^{Thr 389}, phospho ribosomal s6 protein^{Ser240/244} and phospho 4E binding protein 1^{Thr37/45}. Activation of Akt, the canonical mTORC1 activator and AMPK,

the classical inhibitor of mTORC1 were examined by immunoblot assay for phospho-AMPK^{Thr172}.

Autophagy—Autophagy markers were quantified by immunoblots for LC3 lipidation, Beclin1 and p62 expression in protein extracts as reported earlier⁽¹⁹⁾. Autophagy flux was quantified by LC3 lipidation with and without chloroquin as previously described⁽³⁵⁾.

Immunoblots—Immunoblots on protein extracts were performed using protocols established in our laboratories as described earlier. In brief, electrophoresis of 30µg protein was performed on a 8% tris glycine gel except for LC3 lipidation where a 12% gel was used, electrotransferred to PVDF membrane, incubated with primary followed by secondary antibody in the appropriate dilutions, blots developed followed by densitometry of the blots using Image J⁽³²⁾.

Statistical Analyses—All cellular experimental data were generated from at least 3 biologically independent experiments. Animal data were obtained from 8 animals in each group. Data were expressed as mean±SEM and means compared using analysis of variance with Bonferroni posthoc analyses for quantitative data that were normally distributed and the Kruskal-Wallis test for skewed data.

All studies were approved by the Institutional Animal Care and Use Committee at the Cleveland Clinic.

Results

In vitro responses to ammonia withdrawal following hyperammonemia

Myotube diameter was significantly lower in response to hyperammonemia and following withdrawal of ammonia, there was partial recovery by 24 hours and further increase in myotube diameter and area at 48 h withdrawal (Fig. 1, A,B). Global protein synthesis was significantly lower following 24 h of hyperammonemia in myotubes (Fig. 1, C,D). Interestingly, ammonia withdrawal for 24 h resulted in partial recovery of protein synthesis and 48 h withdrawal resulted in a further increase in hyperammonemia induced impaired protein synthesis in myotubes. Expression of myostatin, a molecular mediator of the signaling responses during hyperammonemia was increased and reversed by ammonia withdrawal (Fig. 2, A). Phosphorylation of global protein synthesis regulatory protein eIF2α was increased in response to hyperammonemia and reversed following withdrawal (Fig. 2, B). Consistently, phosphorylation of mTORC1 and its downstream signaling targets, p70S6K and ribosomal S6 protein phosphorylation also recovered nearly completely after 24 h of ammonia withdrawal (Fig. 2, C). Autophagy markers LC3 lipidation was increased and p62 expression decreased during hyperammonemia and these perturbations were reversed by ammonia withdrawal at 24 and 48 hours (Fig. 2, D). Autophagy flux was increased during hyperammonemia and was reversed at 24 and 48 h of ammonia withdrawal (Supporting Fig. 1).

In vivo responses to ammonia lowering therapy in hyperammonemic PCA rat

Body weight, lean body mass and grip strength of PCA and SO rats in the vehicle only and ammonia lowering therapy group are shown in Fig. 3, A, B and Table 1. Following the first week, PCA rats had significantly lower body weight, lean body mass and grip strength compared with SO rats. Lean body mass and grip strength were significantly higher ($p<0.010$) in the PCA rats treated with r-LOLA compared to vehicle alone treated animals. In the SO rats, no significant effect of r-LOLA was noted compared to vehicle treated group. Organ weights in the different groups are shown in Table 2. Liver, testes, spleen and fat mass were significantly lower in the PCA rats treated with vehicle compared to the sham operated pair fed rats. In response to ammonia lowering therapy, only muscle mass was higher than in the untreated PCA rats. No significant differences were noted in other organ weights in response to r-LOLA in the PCA rats.

Muscle mass and fiber diameter were significantly lower ($p<0.001$) in the PCA rats compared to SO in the vehicle treated group (Fig. 3, C). Muscle mass and fiber diameter were greater in the r-LOLA treated PCA rats compared to those treated with vehicle alone ($p<0.01$) but were less than that in the SO rats ($p<0.05$). In the SO rats, r-LOLA did not significantly alter muscle mass or fiber diameter. As reported previously⁽³⁶⁾, PCA rats treated with vehicle had a lower percentage of type II/type I fibers compared to SO rats ($p<0.001$) and treatment with r-LOLA partially reversed this phenotype in the PCA rat muscle fiber type (Fig. 3, D). Reduction in fiber size in the hyperammonemic PCA rat was not accompanied by alterations in muscle fibrosis (Fig. 3, E). Plasma and muscle ammonia concentrations were significantly higher in the vehicle treated PCA rat compared to the other groups (Fig. 4, A, B). r-LOLA treatment resulted in lower plasma and muscle ammonia concentrations. However, muscle ammonia concentrations in the r-LOLA treated PCA rats remained higher than that in the SO rats, showing that 28 days of treatment did not completely reverse hyperammonemia of portosystemic shunting. In the SO rats, r-LOLA did not lower ammonia concentrations. Amino acid concentrations in plasma and muscle tissue are shown in Supporting Tables S1,2. Plasma branched chain amino acids were lower in the PCA than SO rats and in response to r-LOLA there was an increase in BCAA in the PCA rats.

Skeletal muscle fractional and absolute protein synthesis rates in the gastrocnemius muscle were quantified using plasma and intracellular precursor pools and were similar (Fig. 4, C, Supporting Table S3). Both fractional and absolute protein synthesis rates were significantly lower in the PCA compared to SO rats. In response to r-LOLA, protein synthesis rates increased in the PCA rats but did not show any significant change in the SO group. Muscle expression of myostatin was increased in the PCA rats treated with vehicle alone while those treated with ammonia lowering therapy showed reversal of myostatin expression and downstream responses. These observations were consistent with the food efficiency in the various groups (Supporting Table S3).

Signaling responses in the mTORC1, eIF2 α and autophagy pathway components were quantified by immunoblots and showed that impaired mTORC1 signaling increased phosphorylation of eIF2 α and expression of autophagy markers were reversed after ammonia lowering for 28 days (Fig. 5, A–D).

Discussion

Hyperammonemia is a mediator of the liver-muscle axis and impairs proteostasis by impaired protein synthesis and increased autophagy^(16, 17, 19). Ammonia lowering therapies have been used extensively in the treatment of hepatic encephalopathy⁽²⁵⁾ but the effects of this treatment on skeletal muscle proteostasis and muscle mass are not known. The phenotype of lower myotube diameter, metabolic response with lower protein synthesis and altered signaling responses due to hyperammonemia were significantly reversed by ammonia withdrawal in a time dependent manner. The physiological relevance of this was noted in the hyperammonemic PCA rat that had lesser gain in body weight and lean body mass with lower muscle mass and grip strength compared to the SO pair fed control rats. Skeletal muscle from the PCA rats had greater expression of myostatin, increased phosphorylation of eIF2 α , a translational repressor, decreased mTORC1 signaling and higher expression of autophagy markers compared to SO rats. Therapy with r-LOLA lowered plasma and muscle ammonia concentrations *in vivo*. Ammonia lowering resulted in partial recovery of the muscle phenotype in the PCA rat without significant alteration in other organ weights. Finally, cellular signaling responses, increased myostatin expression and autophagy markers and the hyperammonemic stress response in the muscle of the PCA rat were significantly reversed by ammonia lowering therapy.

Even though the adverse effects of hyperammonemia on the skeletal muscle have been reported in the past^(20, 32, 33, 37), we show for the first time that ammonia lowering is a potential therapy for sarcopenia by partially reversing the phenotype, molecular and functional perturbations of hyperammonemia in the PCA rat. In the SO control rats with normal liver function and absence of hyperammonemia, ammonia lowering treatment, expectedly, did not alter the phenotype, muscle protein synthesis and molecular markers. Even though liver and testes weights were lower in the PCA rat, unlike the partial recovery of muscle mass and strength, other organ weights did not change in response to ammonia lowering effects suggesting that the hyperammonemia altered the phenotype and induced a cellular stress response in the skeletal muscle and these effects are reversible with prolonged therapy. These studies are of high clinical relevance because of previous concerns that the reduced muscle mass in cirrhosis, believed to be due to increased muscle ammonia, is not reversed with normalization of ammonia metabolism following liver transplantation^(4, 16). We show that using aggressive ammonia lowering strategy, muscle phenotype and lean body mass improved significantly in the hyperammonemic PCA rat. Our complementary *in vitro* ammonia withdrawal studies in myotubes also showed that the reduced myotube diameter significantly improved but did not completely normalize following ammonia withdrawal. Since ammonia-lowering therapies are clinically approved in patients with cirrhosis, these observations suggest that such treatment has the potential to reverse sarcopenia in cirrhosis.

Our studies also provide novel evidence that ammonia-lowering measures reduce skeletal muscle concentration of ammonia. Our data are consistent with those in previous reports^(20, 36) that muscle and plasma ammonia concentrations were significantly higher in PCA than the pair fed SO rats. In the present study, we used a combination of r-LOLA because the two interventions have different mechanisms of action^(26, 27) and we expected a robust ammonia lowering effect. However, peripheral plasma ammonia concentrations

decreased significantly, but did not reach control values. Muscle ammonia concentrations were also decreased significantly with therapy in the PCA rats but remained higher than that in the muscles of SO control rats. These data are consistent with previous data that ammonia-lowering treatments do not completely normalize blood or cerebrospinal fluid ammonia concentrations^(38–40). It is possible that other measures including ornithine phenylacetate or cell permeable esters of α -ketoglutarate may be more effective ammonia lowering measures but are not available for routine use⁽⁴¹⁾. Even though backflux of ammonia has been reported in the brain⁽²⁹⁾, this is the first report of a reduction in muscle ammonia concentrations in a model of prolonged hyperammonemia following long-term ammonia lowering therapy. Potential mechanisms for incomplete reduction of tissue ammonia concentrations include the continued increased muscle uptake of ammonia in the presence of portosystemic shunting, the need for even longer durations of therapy in the presence of persistent disease or the increased expression of ammonia transporters in the muscle during hyperammonemia⁽⁴²⁾. Even though muscle concentrations of ammonia were not completely reversed following 28 days therapy, it is possible that prolonged therapy may be needed to lower tissue ammonia content and may need studies that focus on ammonia transport function.

Ammonia results in alterations in circulating branched chain amino acid concentrations⁽⁴³⁾. Consistent with previous reports, plasma concentrations of branched chain amino acids were lower in the PCA compared with the SO pair fed rats⁽³³⁾. Similar to our previous report of elevated or unaltered branched chain amino acid concentrations (BCAA) in myotubes during hyperammonemia⁽¹⁷⁾, BCAA concentrations were unaltered or increased in the skeletal muscle of the PCA rat and lowering ammonia reversed this effect. Intracellular amino acid deficiency sensor, GCN2 was activated in the skeletal muscle of the PCA rat even though tissue amino acid concentrations were not reduced in the PCA rat. The mechanism of phosphorylation and activation of GCN2 is suggested to be mediated by ammonia induced mitochondrial sequestration of leucine⁽¹⁷⁾. Even though ammonia lowering did alter plasma amino acids, there was no significant effect on the muscle concentrations of amino acids. Interestingly, despite no alteration in muscle amino acid concentrations, GCN2 activation and phosphorylation of eIF2 α were partially reversed by ammonia lowering. These observations suggest that reduction in ammonia prevents amino acid utilization for ammonia detoxification⁽⁴³⁾ or alters subcellular localization of amino acids with consequent sensing of amino acid adequacy in the cytoplasmic compartment. Whether hyperammonemia or ammonia lowering alters the subcellular localization of GCN2 is currently unknown.

Expression of myostatin, a TGF β superfamily member and known inhibitor of muscle mass, was increased in both myotubes and in the muscle from the hyperammonemic PCA rat. These observations were consistent with previous reports by us that hyperammonemia transcriptionally upregulates myostatin⁽¹⁶⁾. The novel observations in this study are that ammonia induced upregulation of myostatin and the consequent decreased protein synthesis are partially reversible *in vitro* and *in vivo*. Increased myostatin expression was not fully reversed by ammonia-lowering therapy that may be due to incomplete reduction in muscle ammonia concentrations or additional ammonia-independent mechanisms of myostatin regulation *in vivo*⁽²⁸⁾. Targeting ammonia alone may also not be adequate to reverse myostatin expression, since our *in vitro* data suggest that after 48 h of ammonia withdrawal

there is nearly complete reversal of the molecular perturbations but *in vivo* ammonia reduction does not completely reverse these alterations. Another potential reason for the difference between the *in vitro* and *in vivo* responses to ammonia lowering is that there is continued ammonia generation *in vivo* with impaired disposal due to the underlying liver disease. In this situation, the skeletal muscle remains a major organ for ammonia disposal and consequent molecular responses. Furthermore, our strategy focused on the classical methods to lower gut bacterial ammoniogenesis and the use of LOLA. Even though we used 28 days of ammonia lowering therapy, longer-term and alternative ammonia disposal treatments may be necessary for complete normalization of muscle ammonia concentrations.

In the present studies, in addition to reversal of GCN2/eIF2 α phosphorylation *in vivo*, mTORC1 signaling was also reversed following ammonia withdrawal in myotubes. Similar responses were observed *in vivo* in the skeletal muscle of the PCA rat following 4 weeks of ammonia lowering therapy. However, the response *in vitro* to ammonia withdrawal was more robust compared with those in the PCA rats. These observations suggest that incomplete reduction in ammonia concentrations *in vivo* result in only partial reversal of mTORC1 or that other factors besides hyperammonemia contribute to impaired mTORC1 signaling. The downstream molecular consequences of reduced myostatin expression are similar to those reported by us and others^(20, 21). We also observed that expression of autophagy markers was increased in response to hyperammonemia as has been previously reported^(18, 19) and ammonia withdrawal reversed the elevated autophagy markers (LC3 lipidation, lower p62 expression) both *in vitro* and *in vivo* unlike the incomplete reversal in reduced protein synthesis. These data show that even though ammonia impairs muscle proteostasis, protein synthesis and autophagy markers and flux respond differently to ammonia withdrawal or lowering, and the mechanisms of these needs to be evaluated further.

Our observations are consistent with previous reports of decreased muscle protein synthesis and a GCN2 dependent hyperammonemic cellular stress response during hyperammonemia^(17, 37). However, there are no studies on muscle proteostasis following ammonia withdrawal to determine if these effects are indeed reversible. This is particularly relevant, because following liver transplantation, even though ammonia metabolism in the graft is normal⁽⁴⁾, sarcopenia does not reverse suggesting that ammonia induced perturbations may not be reversible. However, our data in an *in vitro* and *in vivo* model show that ammonia lowering as a strategy to reverse sarcopenia has the potential for rapid clinical translation in patients with liver disease.

We provide for the first time, evidence that ammonia lowering strategies in an established preclinical model of sarcopenia in cirrhosis with portosystemic shunting results in partial reversal of muscle loss, molecular perturbations and recovery of fractional and absolute protein synthesis rates. These data lay the foundation for clinical translational studies in the therapy of muscle loss in liver disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations

ASR	absolute synthesis rate
BCAA	branched chain amino acids
DMEM	Dulbecco's modified Engle eIF eukaryotic initiation factor
FSR	fractional protein synthesis rate
GCN	general control non-derepressible
LOLA	L ornithine L aspartate
mTORC1	mammalian target of rapamycin complex 1
PCA	portacaval anastomosis
r-LOLA	rifaximin-LOLA
SO	sham operated pair fed
TGF	transforming growth factor
TOBEC	total body electrical conductivity.

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Fig. 1. Hyperammonemia induced reduction in myotube size and protein synthesis reversed by ammonia withdrawal

A, B. Reduction in myotube diameter reversed following ammonia withdrawal.

Representative photomicrographs and quantification of myotube diameter in C2C12 myotubes treated with 10mM ammonium acetate (AmAc), following withdrawal of ammonia and untreated controls (CTL). Lower myotube diameter was partially at 24 h and completely reversed at 48 h after withdrawal of ammonia. All experiments from at least 100 myotubes from 4 independent plates of cells. Data expressed as Mean $\pm$ SD. * $p < 0.05$, *** $p < 0.001$ vs. untreated control.

C, D. Partial recovery of protein synthesis in myotube following ammonia withdrawal.

Representative immunoblots and densitometry from at least 3 biological replicates of puromycin incorporation in C2C12 myotubes treated with 10mM ammonium acetate (AmAc), following withdrawal of ammonium acetate and untreated controls. Data expressed as Mean $\pm$ SD from experiments in at least 3 biological replicates. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Fig. 2. Recovery of signaling responses following ammonia withdrawal in myotubes
A. Representative immunoblots and densitometry of myostatin, phospho AMPK and its target, ACC in C2C12 myotubes treated with 10mM ammonium acetate (AmAc), and following replacement with ammonia free differentiation **B.** Representative immunoblots and densitometry of eIF2 α kinase phospho GCN2 and phosphorylated eIF 2 α in C2C12 myotubes during hyperammonemia and following replacement of medium with ammonia free differentiation medium for 24 h and 48 h. Data show near complete reversal of ammonia induced alterations in activation of GCN2 and phosphorylation of eIF2 α .

C. Representative immunoblots and densitometry of mTORC1 signaling (phosphorylation of mTORC1, P70S6 kinase, ribosomal S6 protein and 4EBP1). Data show that ammonia impairs mTORC1 activation and signaling that is partially reversed after ammonia withdrawal.

D. Representative immunoblots and densitometry show that increased LC3 lipidation and lower P62 expression following exposure to 10mM AmAc were reversed after 24 h and 48 h of ammonia withdrawal.

All experiments were done in 3 biological replicates, all data shown as mean $\pm$ SD. * $p<0.05$, *** $p<0.001$ vs. untreated controls. † $p<0.05$ vs. 24 AmAc; ¶ $p<0.05$ vs. 24h withdrawal group.

Fig. 3. Lowering ammonia in the portacaval anastomosis rat reverses sarcopenia

A,B. Change in body weight and lean body mass in the portacaval anastomosis (PCA) and sham operated pair fed (SO) rats treated with vehicle alone or ammonia lowering therapy with L-ornithine-L-aspartate and rifaximin (r-LOLA). Significantly lower whole body mass in the PCA rats treated with vehicle alone was partially reversed after 4 weeks of r-LOLA therapy while no effect was observed in the sham operated (SO) rats. *** p<0.001, ** p<0.01 vs. SO rats; ¶ p<0.05 vs. PCA r-LOLA; Ψ p<0.01, Φ p<0.001 vs. PCA r-LOLA.

C. Representative photomicrographs of hematoxylin eosin stained cryosections of gastrocnemius muscle of from PCA and sham rats treated with vehicle alone or ammonia lowering therapy with r-LOLA. Mean minimum internal diameter and mean area of the muscle fibers expressed as percentage of sham operated controls treated with vehicle alone showed reduction in muscle fiber size that was partially reversed by r-LOLA treatment for 4 weeks. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

D. Representative photomicrographs of cryosections of gastrocnemius muscle showing fiber type identification using ATPase staining at pH 10. Lower ratio of type II/type I fiber numbers was observed in the PCA rats treated with vehicle compared to sham operated rats. r-LOLA treatment for 4 week reversed the reduction in typeII/type I ratio. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

E. Representative photomicrographs of cryosections of gastrocnemius muscle from PCA and sham operated rats treated with vehicle alone and r-LOLA showed no increase in fibrosis. All experiments in $n=8$ animals in each group. All histomorphometry from at least 50 fibers per muscle from each animal in each group. All data mean $\pm$ SD. r-LOLA: rifaximin-L-ornithine L aspartate orally for 28 days.

Fig. 4. Therapy effectively lowered plasma and muscle ammonia concentrations and reversed impaired muscle protein synthesis in PCA rat

A. Elevated plasma ammonia in the vehicle treated PCA rats lowered after r-LOLA therapy. Non-significant reduction in plasma ammonia in the sham operated rats treated with r-LOLA.

B. Elevated skeletal muscle ammonia in the vehicle treated PCA rats lowered but remains higher than that in the sham operated rat gastrocnemius muscle.

C. Fractional synthesis rate in the vehicle treated PCA rats significantly lower than that in sham operated rats. Ammonia lowering therapy with r-LOLA partially reversed this reduction in protein synthesis rate.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. $n=8$ animals in each group. All data mean $\pm$ SD. r-LOLA: rifaximin-L-ornithine L aspartate orally for 28 days.

Fig. 5. *In vivo* molecular responses to hyperammonemia partially reversed after ammonia lowering therapy

Representative immunoblots and densitometry of proteins regulating proteostasis in the gastrocnemius muscle of the hyperammonemic PCA or sham operated pair fed (sham) rats treated with vehicle or ammonia lowering therapy with r-LOLA for 28 days.

A. Representative immunoblots and densitometry of the expression of myostatin, phospho AMPK and its target ACC significantly higher in the hyperammonemic PCA rats treated with vehicle compared to sham rats. Ammonia lowering therapy (r-LOLA) resulted in partial reversal of these perturbations in the PCA rat.

B. Representative immunoblots and densitometry of amino acid deficiency sensor and eIF2 α kinase, GCN2 and its target eIF2 show increased expression in the hyperammonemic PCA rats treated with vehicle alone compared to the other groups. Ammonia lowering therapy (r-LOLA) partially reversed the increased phosphorylation of GCN2 and eIF2 α in the gastrocnemius muscle of the PCA rat.

C. Representative immunoblots and densitometry showed impaired mTORC1 activation and signaling in the PCA rats treated with vehicle compared with sham operated control rats. Ammonia lowering therapy partially reversed the molecular perturbations in mTORC1 signaling.

D. Representative immunoblots and densitometry showed increased autophagy markers, LC3 lipidation and lower P62 expression in the gastrocnemius muscle from the hyperammonemic PCA rat treated with vehicle compared with the sham operated control rats. Ammonia lowering therapy partially reversed these alterations. All data expressed as mean $\pm$ SD from 6 rats in each group. * $p<0.05$; ** $p<0.01$; *** $p<0.001$. r-LOLA: rifaximin-L-ornithine L aspartate orally for 28 days.

Table 1

Serial changes in grip strength

	Sham +Vehicle	PCA+vehicle	Sham+r-LOLA	PCA +r-LOLA
Number	8	8	8	8
0 week	1.35±0.05	0.64±0.04 [§]	1.24±0.05	0.62±0.03 [§]
1 Week	1.86±0.05	0.87±0.03 [§]	1.86±0.04	1.12±0.04 [§]
2 Week	2.24±0.06	0.95±0.03 [§]	2.25±0.06	1.71±0.07 ^{§,¶}
3 Week	2.94±0.07	1.32±0.03 [§]	2.93±0.07	2.22±0.05 ^{§,¶}
4 Week	3.56±0.06	1.54±0.03 [§]	3.85±0.07	2.90±0.04 ^{§,¶}

All values are in lbs and data expressed as mean±SD

[§]p<0.001 vs. Sham+vehicle;[¶]p<0.01 vs. PCA + vehicle. r-LOLA rifaximin + LOLA for 28 days.

Table 2

Organ weights at 4 weeks of treatment

	Sham+vehicle	PCA +vehicle	Sham+r-LOLA	PCA+r-LOLA
Number	8	8	8	8
Gastroc	3.53±0.34	2.28±0.62 [§]	3.66±0.28	2.94±0.42 [¶] ^Φ
EDL	1.12±0.14	0.73±0.19 [§]	1.31±0.10	1.06±0.25 ^Φ
Liver	20.71±3.53	7.49±2.07 [§]	18.85±2.4	6.96±2.04 [§]
Spleen	0.99±0.16	0.78±0.18 [¶]	1.31±0.32	1.02±0.33
Heart	1.86±0.31	1.50±0.45	1.72±0.20	1.60±0.19
Testis	1.97±0.12	0.99±0.43 [§]	2.07±0.23	1.04±0.36 [§]
Fat	2.99±0.71	1.59±0.36 [§]	3.27±0.96	1.86±0.42 [§]

All values mean±SD.

[§] p<0.001,[¶] p<0.01 vs. Sham+vehicle;^Φ p<0.01 PCA +vehicle vs. PCA+r-LOLA. r-LOLA rifaximin + LOLA for 28 days.