Hyperammonemia-mediated autophagy in skeletal muscle contributes to sarcopenia of cirrhosis

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Hyperammonemia-mediated autophagy in skeletal muscle contributes to sarcopenia of cirrhosis. Am J Physiol Endocrinol Metab 303: E983–E993, 2012. First published August 14, 2012; doi:10.1152/ajpendo.00183.2012.—Hyperammonemia and sarcopenia (loss of skeletal muscle) are consistent abnormalities in cirrhosis and portosystemic shunting. We have shown that muscle ubiquitin-proteasome components are not increased with hyperammonemia despite sarcopenia. This suggests that an alternative mechanism of proteolysis contributes to sarcopenia in cirrhosis. We hypothesized that autophagy could be this alternative pathway since we observed increases in classic autophagy markers, increased LC3 lipidation, beclin-1 expression, and p62 degradation in immunoblots of skeletal muscle protein in cirrhotic patients. We observed similar changes in these autophagy markers in the portacaval anastomosis (PCA) rat model. To determine the mechanistic relationship between hyperammonemia and autophagy, we exposed murine C57BL/6J myotubes to ammonium acetate. Significant increases in LC3 lipidation, beclin-1 expression, and p62 degradation occurred by 1 h, whereas autophagy gene expression (LC3, Atg5, Atg7, beclin-1) increased at 24 h. C2C12 cells stably expressing GFP-LC3 or GFP-mCherry-LC3 constructs showed increased formation of mature autophagosomes supported by electron microscopic studies. Hyperammonemia also increased autophagic flux in mice, as quantified by an in vivo autophagometer. Because hyperammonemia induces nitration of proteins in astrocytes, we quantified global muscle protein nitration in cirrhotic patients, in the PCA rat, and in C2C12 cells treated with ammonium acetate. Increased protein nitration was observed in all of these systems. Furthermore, colocalization of nitrated proteins with GFP-LC3-positive puncta in hyperammonemic C2C12 cells suggested that autophagy is involved in degradation of nitrated proteins. These observations show that increased skeletal muscle autophagy in cirrhosis is mediated by hyperammonemia and may contribute to sarcopenia of cirrhosis.

Potential mediators of sarcopenia in cirrhosis is hyperammonemia (13). Since the liver is the major organ responsible for ammonia detoxification, hepatocellular dysfunction and portosystemic shunting in cirrhosis contribute to elevated blood ammonia concentrations (35). Our preliminary studies have shown that hyperammonemia-induced, myostatin-mediated impaired protein synthesis is a consistent abnormality in animal models and in vitro systems (10, 13a). However, a reduction in skeletal muscle protein synthesis alone is not sufficient to account for continued reduction in muscle mass in cirrhosis, and an increase in proteolysis is necessary (18). Since components of the ubiquitin-proteasome proteolysis pathway are not altered in the portacaval anastomosis (PCA) rat with sarcopenia (9–11), there must be alternative mechanisms of protein breakdown contributing to the loss of muscle mass in cirrhosis (28, 29). In this regard, the lysosomal proteolytic pathway of autophagy is a highly conserved process. It is responsible for controlled degradation of cytoplasmic components, including damaged organelles/toxic protein aggregates, as well as providing essential nutrients during starvation and stress to maintain cellular homeostasis (2). Since cirrhosis is a state of accelerated starvation (6, 47), enhanced muscle autophagy may serve as a source of essential amino acids for critical cellular function.

In addition to providing essential nutrients, autophagy plays a critical role in the removal of damaged and abnormal proteins (21). Hyperammonemia has been identified recently to be a potential inducer of autophagy (7, 15, 16, 30), and significant hyperammonemia is a consistent abnormality in cirrhosis (34, 35). Hyperammonemia induces nitration of proteins in astrocytes (20, 41), and nitration has been reported to cause structural changes and impair protein function (1, 3, 39, 46). Therefore, increased autophagy may be a response to degrade these abnormal proteins in the skeletal muscle during hyperammonemia of cirrhosis. Autophagy is dysregulated in pathophysiological states, and an understanding of the mechanisms regulating this pathway can provide novel therapeutic options (21). In this study, we examined the induction of autophagy by hyperammonemia as a potential cause of skeletal muscle loss in cirrhosis.

MATERIALS AND METHODS

Reagents. Antibodies were obtained as follows: LC3 (Novus Biological, Littleton, CO), p62 (Progen Biotechnical, Heidelberg, Germany), beclin-1 (Cell Signaling Technology, Danvers, MA), nitrotyrosine (Millipore, Billerica, MA), β-actin, and α-tubulin, as well as...
the secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Ammonium acetate, ammonium chloride, probenecid, potassium iodophore nigericin, tyrsoine, nitrotyrosine, and other reagents for making buffers were obtained from Sigma-Aldrich (St. Louis, MO). Free 2',7'-biscarboxyl-ethyl-5(6)-carboxyfluorescein (BCECF) and BCECF acetomethyl ester (BCECF-AM) were obtained from Life Technologies (Grand Island, NY).

Human studies. Autophagy was studied in human skeletal muscle from patients with cirrhosis undergoing liver transplantation (n = 13). All patients had histologically confirmed cirrhosis in the explanted liver. Subjects undergoing elective abdominal surgery without disorders or medications that affect skeletal muscle synthesis or breakdown formed the control group (n = 13; hernia repair = 3; donors for liver transplantation, n = 10). The clinical and biochemical characteristics of the two groups are shown in Table 1. Muscle mass was measured by quantifying psoas and paraspinal and abdominal wall areas on CT scans of the abdomen at L4 vertebra (48). This was significantly lower (P < 0.01) in cirrhotics compared with that in control subjects. The studies were approved by the Institutional Review Board of the Cleveland Clinic, and written informed consent was obtained from all subjects.

The rectus abdominis muscle was obtained from these subjects at the initiation of surgery, flash-frozen in liquid nitrogen, and stored at −80°C for subsequent assays.

**Table 1. Clinical and demographic features of subjects**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control</th>
<th>Cirrhosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI, kg/m²</td>
<td>29.3</td>
<td>6.5</td>
</tr>
<tr>
<td>Sex (males/females)</td>
<td>7/6</td>
<td>7/6</td>
</tr>
<tr>
<td>Age, yr</td>
<td>51.2 ± 12.1</td>
<td>54.0 ± 10.3</td>
</tr>
<tr>
<td>Sex (males/females)</td>
<td>7/6</td>
<td>7/6</td>
</tr>
<tr>
<td>Age, yr</td>
<td>51.2 ± 12.1</td>
<td>54.0 ± 10.3</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>29.3 ± 6.5</td>
<td>29.7 ± 4</td>
</tr>
<tr>
<td>Bilirubin, mg/dl</td>
<td>0.5 ± 0.2</td>
<td>11.0 ± 17.8***</td>
</tr>
<tr>
<td>Alanine aminotransferase, IU/dl</td>
<td>28.5 ± 20.5</td>
<td>75.6 ± 11.9***</td>
</tr>
<tr>
<td>Aspartate aminotransferase, IU/dl</td>
<td>35.6 ± 21.9</td>
<td>134.0 ± 240.1***</td>
</tr>
<tr>
<td>Serum creatinine, mg/dl</td>
<td>1.0 ± 0.3</td>
<td>1.4 ± 0.4**</td>
</tr>
<tr>
<td>Blood urea nitrogen, mg/dl</td>
<td>13.5 ± 4.4</td>
<td>24.8 ± 19.4*</td>
</tr>
<tr>
<td>International normalized ratio</td>
<td>1.2 ± 0.5</td>
<td>1.6 ± 0.5**</td>
</tr>
<tr>
<td>Serum albumin, g/dl</td>
<td>3.9 ± 0.6</td>
<td>3.4 ± 0.9***</td>
</tr>
</tbody>
</table>

Values are means ± SE. BMI, body mass index. *P < 0.05; **P < 0.01; ***P < 0.001, cirrhosis vs. controls.

**DNA extraction.** Muscle tissue was homogenized, followed by centrifugation at 12,000 g for 5 min at 4°C. The supernatant containing the protein extract was transferred into a new microcentrifuge tube, and protein content was quantified using the bicinchoninic acid assay (Thermo Fisher Scientific, Aliquippa, PA) and stored at −80°C for subsequent assays.

**Western blots.** Immunoblots were performed as described previously (9). In brief, protein samples were diluted in SDS-PAGE sample buffer and denatured, followed by loading of equal amounts of protein in a 4–12% gradient gel. Following electrophoresis, the proteins were electrotransferred onto polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA) that were blocked using 5% nonfat milk in Tris-buffered saline (TBS). The membranes were incubated overnight at 4°C in primary antibodies in TBS with 0.5% nonfat milk in TBS with 0.5% nonfat milk (p62, α-tubulin) and α-tubulin as loading control were performed, followed by densitometry for quantification of the bands. These studies were performed at Washington University (St. Louis, MO), as described previously (26).

**Cell culture.** Murine C2C12 cell lines were obtained from ATCC (Manassas, VA) and grown to confluence in proliferation medium consisting of Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal calf serum. After confluence, the medium was changed to differentiation medium (DMEM with 2% horse serum) for the cells to differentiate. Following 48 h of differentiation, cells were exposed to ammonium acetate (10 mM) for various time points to 24 h. Control and treated cells were incubated with 0.4% trypan blue in sterile phosphate-buffered saline (PBS) and immediate counting of cells. Since the trypan blue exclusion suffers from the limitations of subjectivity between multiple shades of blue or blue-gray as well as overestimation of cell viability by this method, a fluorescent cell viability assay (CellTiter-Blue cell viability assay; Promega, Madison, WI) was also performed using the manufacturer protocol prior to the detailed molecular studies. This provided a homogeneous, fluorometric method for estimating viable cells. Viable cells reduce a redox dye (resazurin) into a fluorescent end product (resorufin), whereas nonviable cells do not reduce the indicator dye and fail to generate a fluorescent signal. Since the trypan blue and the CellTiter blue use different chemistries to determine cell viability, both of these methods were used to show high cell viability in response to hyperammonemia.

**Muscle tissue protein and RNA extraction.** Protocols for muscle protein and total RNA extraction have been established in our laboratory and described earlier (9, 11). In brief, for total RNA extraction, ~15 mg of the frozen muscle sample was used. RNA was isolated by using RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol, using chloroform extraction. RNA concentration was determined using the NanoDrop 1,000 UV/Vis Spectrophotometer (Thermo Scientific, Rockford, IL). The quality of RNA was evaluated by gel electrophoresis on a formaldehyde RNA gel.

Total muscle protein was extracted from a precisely weighed amount (~40 mg) of frozen skeletal muscle samples. Samples were added to the Lysing Matrix D (MP Biomedicals, Solon, OH) tubes with ice-cold RIPA buffer (Thermo Scientific) supplemented with protease and phosphatase inhibitors (Thermo Scientific) for extracting protein. Using the FastPrep 120 (Q-BIOgene, Irvine, CA), the tissue was homogenized, followed by centrifugation at 12,000 g for 5 min at 4°C. The supernatant containing the protein extract was transferred into a new microcentrifuge tube, and protein content was quantified using the bicinchoninic acid assay (Thermo Fisher Scientific), aliquotted, and stored at −80°C for subsequent assays.

**20S proteasome activity assay.** The 20S proteasome activity assay was performed on Stratagene Mx 3000P (Stratagene, La Jolla, CA), using a SYBR protocol on the fluorescence temperature cycler by methods as described previously (9, 11). Relative differences were normalized to the expression of β-actin. Expression of autophagy genes was quantified, and the primer sequences are shown in Table 2. Real-time PCR products were then separated by gel electrophoresis to confirm specific product presence and size.

**Quantitative real-time PCR.** Real-time polymerase chain reaction for quantification of mRNA was performed on a Stratagene Mx 3000P (Stratagene, La Jolla, CA), using a SYBR protocol on the fluorescence temperature cycler by methods as described previously (9, 11). Relative differences were normalized to the expression of β-actin. Expression of autophagy genes was quantified, and the primer sequences are shown in Table 2. Real-time PCR products were then separated by gel electrophoresis to confirm specific product presence and size.
Table 2. Primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession No.</th>
<th>Upper (5‘-3’)</th>
<th>Lower (5‘-3’)</th>
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<td>TGGAAATCTCGAATGAGCTG</td>
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<td>Proteasome C5</td>
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<td>Murlf1</td>
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<tr>
<td>Atrogin</td>
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<td>ULK2</td>
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<td>Beclin-1</td>
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<td>LC3b</td>
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<tr>
<td>β-Actin</td>
<td>NM_007393.5</td>
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</table>

Murlf1, muscle RING finger 1.

Based on detection of the fluorophore 7-amino-4-methylcoumarin (AMC) after cleavage from the labeled substrate LLVY-AMC was used for these studies (10). Enzyme activity of the 20S proteasome was expressed as relative fluorescence units per microgram of protein. For C2C12 cells, whole cell lysates were prepared according to the manufacturer’s instructions, and the assay was performed with the fluorescence units normalized to the protein content. All experiments were performed in triplicate, and the values are expressed as means ± SD.

*Generation of stably transfected C2C12 cells.* Stable lines expressing GFP-LC3 and GFP-mCherry-LC3 were generated by retrovirus and lentivirus transduction, respectively, using protocols approved by the Institutional Biosafety Committee at the Cleveland Clinic (43). The GFP-mCherry-LC3 plasmid was a kind gift from Dr. Jayanta Debnath (University of California San Francisco). In brief, the constructs were packaged in human embryonic kidney (HEK)-293T cells that were transfected with GFP-mCherry-LC3 constructs and pCMV plasmid in a 3:1 ratio using the Fugene transfection reagent. The GFP-mCherry-LC3 tandem reporter C2C12 cells on glass slides grown in 70 – 80% monolayers of stably transfected GFP-LC3 or GFP-mCherry-LC3 were generated by retrovirus transduction, respectively, using protocols approved by the Institutional Biosafety Committee at the Cleveland Clinic (43). GFP-mCherry-LC3 after treatment were fixed with 4% paraformaldehyde at room temperature for 30 min. After another 3 PBS washes, samples were mounted in Vectashield mounting medium for fluorescence with 4.6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). Images were acquired on a Leica TCS SP2 confocal laser scanning microscope. The 488-nm line of an argon/argon-krypton laser and the 561-nm line of a solid-state laser were used to excite the samples. Cells were imaged through a ×40/1.4 NA oil immersion objective. Images were collected and saved using Leica Confocal software and exported to Microsoft Picture Manager for digital processing.

*Analysis of GFP-LC3 puncta.* The GFP-LC3 puncta were counted and expressed as number of cells with >5 green puncta/cell, as described previously (22). GFP-LC3 puncta were scored in z-stack overlays from at least four separate fields with at least 100 nuclei, and autophagosome analysis was performed using a customized virtual basic Image-Pro macro.

*Electron microscopy.* C2C12 cells in 2% horse serum DMEM were treated with 10 mM ammonium acetate for 6 h or ammonium acetate for 6 h with 100 μM chloroquine to block lysosomal degradation and a combination of chloroquine and ammonium acetate to examine autophagic flux. The results were compared with control untreated cells. After appropriate washing, cells were fixed in 2.5% glutaraldehyde for 1 h. After en bloc staining and ethanol dehydration, samples were embedded with eponate 12 medium. Sections (85 nm) were cut using a diamond knife, followed by double staining using uranyl acetate and lead citrate. Electron microscopy was performed at the Imaging Core at the Lerner Research Institute at the Cleveland Clinic on a Philips CM12 electron microscope (FEI, Hillsboro, OR) operated at 10 kV. The pH-sensitive dye BCECF was used for the recording of intracellular pH, using a spectrofluorometric method that measures the fluorescence intensity ratio of intracellular dye (24, 42). In brief, after differentiation for 48 h in six-well plates, cells were loaded with BCECF-AM (1 μM in DMSO). Probenecid, an inhibitor of organic anion transporter, was added to a final concentration of 2.5 mM to reduce leakage of free BCECF from the intracellular compartment. Cells were incubated for 30 min at 37°C with the dye in HEPES buffered balanced salt solution (130 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1.5 mM CaCl2, 25 mM HEPES, 5 mM glucose, 0.1% bovine serum albumin, pH 7.5, ~290 osmolality). Cells were then washed with calcium-balanced salt solution buffer,
and the ratio of fluorescence with 440 (isobestic point for BCECF) and 480 nm excitation, 530-nm emission, was corrected for background fluorescence obtained from the cells without dye. Measurements were done at 37°C on a BioTek Synergy II plate reader (BioTek US, Winooski, VT). Response to ammonium acetate was compared with untreated control cells. After 20 min of incubation, cells were then washed with calcium-balanced salt solution to remove the extracellular ammonium acetate, and the fluorescence was recorded again for 10 min to demonstrate that removal of extracellular ammonium acetate resulted in the intracellular release of ammonia that diffuses out with subsequent reduction in intracellular pH. This showed that there was intracellular transport of ammonium ion. The calibration of BCECF was determined using the high K+ nigericin technique. Briefly, cells loaded with BCECF were transferred into calibration solutions containing 120 mM KCl, 1 mM MgCl2, 1.5 mM CaCl2, 30 mM HEPES, and 10 mM nigericin (a K/H antiporter), with the extracellular pH adjusted to 7.4, 8.3, and 6.7 and the fluorescence read at 37°C for 4 min each. Additionally, 10 μM free BCECF in PBS was used to determine the fluorescence of buffer solutions ranging from pH 5.8 to pH 8.0. This was used to show that the extracellular pH measurements were precise to values of ±5%. Simultaneously, differentiated C2C12 cells were exposed to 10 mM ammonium chloride to demonstrate the anticipated changes in intracellular pH (4). All experiments were done in quadruplicate.

Quantification of nitrotyrosine and tyrosine in C2C12 cells. Nitrotyrosine content in control and ammonium acetate-treated differentiated C2C12 cells was quantified by stable isotope dilution, LC-MS/MS, as described earlier (5). In brief, cells were washed with ice-cold PBS and harvested by trypsinization. After sonication of the cells, protein was precipitated and protein-bound 3-nitrotyrosine content in the lysates analyzed by HPLC with an on-line electrospray ionization tandem mass spectrometry (LC/ESI/MS/MS) on a triple quadrupole mass spectrometer (API 365; Applied Biosystems, Foster City, CA) with an Ionics EP 10+ upgrade (Ionics; Concord, Ontario, CA) interfaced to a Cosehie Technologies (Franklin, MA) Aria LX Series HPLC multiplexing system. Synthetic [13C6]-labeled standard was used as an internal standard to quantify the natural abundance 3-nitrotyrosine. Universal label tyrosine [13C9,15N1] was used to quantify tyrosine and to monitor for the presence of potential artificial nitration (which was <5% total), as monitored by the universally labeled 3-nitrotyrosine isotopologues. Results presented are normalized to the content of the precursor amino acid tyrosine measured during the same injection.

Statistical analysis. All data are expressed as means ± SD unless specified. All experiments were done in triplicate. Qualitative variables were compared by the Chi squared test and quantitative and rating variables compared using the analysis of variance with SPSS 20.0 (IBM, Armonk, NY).

RESULTS

Autophagy is increased in skeletal muscle from cirrhotic patients. As mentioned earlier, since impaired protein synthesis alone is not adequate to result in a reduction in muscle mass, an increase in proteolysis is necessary for sarcopenia. An increase in ubiquitin-proteasome-mediated proteolysis has been reported in other disorders with significant loss of muscle mass (18). In the present study, components of the ubiquitin-proteasome pathway were quantified by real-time PCR (Fig. 1A), and no significant differences (P > 0.1) were observed between well-characterized patients with cirrhosis and controls. The 20S proteasome activity assay also did not show a difference (P > 0.1) between cirrhotics and controls (Fig. 1B). Since the

Fig. 1. Skeletal muscle autophagy but not proteasome-mediated proteolysis is increased in cirrhosis and portosystemic shunting: A: real-time PCR quantification of critical components of the ubiquitin-proteasome pathway in the rectus abdominis skeletal muscle of patients with cirrhosis (n = 13) compared with controls (n = 13). No significant differences were observed between the 2 groups. B: proteasome 20S activity assay in skeletal muscle lysate from cirrhotics (n = 10) and controls (n = 10) showed no significant differences between the 2 groups. C: representative immunoblots performed on total protein extracted from skeletal muscle biopsies from cirrhotic patients (n = 13) and control subjects of p62, beclin-1, and LC3 lipidation (each lane represents a separate subject). D: densitometry with error bars of the blots normalized to β-actin. Increased LC3 lipidation, p62 degradation, and beclin-1 expression in cirrhosis compared with controls. *P < 0.01. E: representative immunoblots performed on total protein extracted from gastrocnemius muscle of portacaval anastomosis (PCA) rat and pair-fed sham-operated control rats showed increased LC3 lipidation and p62 degradation in sham control and PCA rats (each lane is a separate animal). F: densitometry with error bars for the blots normalized to β-actin. **P < 0.01 PCA compared with sham controls.
ubiquitin-proteasome pathway was unaltered in cirrhosis, we examined skeletal muscle autophagic flux since it is another proteolytic pathway that is active during stress and starvation (2). Key components of this pathway include a component (beclin-1) of the phosphatidylinositol 3-kinase complex, a cytosolic protein (LC3-I) that is lipidated (LC3 II) to facilitate autophagosome membrane formation, and p62/SQSTM1 that targets cargo to the autophagosome for degradation. We measured autophagy using standard readouts, including LC3 lipidation, beclin-1 expression, and p62 degradation in cirrhotic patients. Expression of these autophagy markers was enhanced in the skeletal muscle of cirrhotic patients undergoing liver transplantation but not in noncirrhotic control subjects (Fig. 1C). Densitometric analysis from cirrhotics (n = 13) and controls (n = 13) showed significant increases in both LC3-II levels and the ratio of LC3-II/LC3-I, thus supporting increased autophagy in the muscle of patients with cirrhosis (Fig. 1D).

**Hyperammonemia contributes to enhanced autophagy in PCA rat model.** The PCA rat is a well-characterized model of sarcopenia in cirrhosis with portosystemic shunting and is associated with hyperammonemia (9, 10, 12, 13). Plasma concentrations of ammonia were significantly (P < 0.01) elevated in the PCA compared with the sham rats (239.4 ± 63.1 μmol/l in PCA and 79.4 ± 36.2 μmol/l in sham-operated controls). Previously, we have reported unaltered ubiquitin-proteasome-mediated proteolysis in this model despite loss of muscle mass (10). Analysis of autophagy markers in the muscle of PCA and sham control rats showed significant increases in LC3-II lipidation and beclin-1 expression as well as p62 degradation (Fig. 1, E and F). Together with our studies in the muscle from human cirrhosis, these data support the idea that hyperammonemia mediates an increase in autophagy, contributing to the loss of muscle mass in cirrhosis.

**Hyperammonemia induces autophagy in C2C12 myotubes.** Cell viability quantified by the fluorescent assay (Promega) as well as by trypan blue exclusion consistently showed >94% cell viability in response to ammonium acetate (Fig. 2A). Similar to our observations in the muscle from human cirrhosis (Fig. 1B) and published data in the PCA rat (10), hyperammonemia did not alter the proteasome activity in the murine myotubes (Fig. 2B). To demonstrate that hyperammonemia mediates increased autophagy, C2C12 myotubes were treated with ammonium acetate for different times. A significant increase in beclin-1 expression and LC3 lipidation was observed by 1 h of treatment, and these were associated with p62 degradation (Fig. 2C). Densitometric analysis showed a significant increase in the LC3-II/LC3-I ratio in response to hyperammonemia (Fig. 2D). Further analysis examined the formation of LC3+ vesicles in response to ammonium acetate in C2C12 cell lines stably expressing GFP-LC3. Hyperammonemia induced the time-dependent formation of GFP-LC3 positive vesicles (autophagosomes) in these cells, which peaked at 6 h (P = 0.03) (Fig. 2, E and F). To complement these observations, we demonstrated increased autophagy in C2C12 myotubes stably expressing the GFP-mCherry-LC3 tandem reporter. In these assays, the formation and maturation of autophagosomes can be monitored by the formation of dual fluorescent vesicles (autophagosomes), which mature through fusion with the lysosome. As the GFP-mCherry+ autophagosome matures and becomes acidified it leads to quenching of the GFP fluorescence, resulting in mCherry+ vesicles (Fig. 2G).

Consistent with this process, we observed an initial increase in green puncta due to the formation of the autophagosome that transitioned to yellow (combination of red and green) and finally the red puncta, demonstrating the formation of the autophagolysosome during hyperammonemia (Fig. 2H).

Finally, to further demonstrate formation and maturation of the autophagosomes in response to hyperammonemia and increased autophagic flux, electron microscopy was carried out. We observed formation of double-lamellar vesicles representing the autophagosomes in C2C12 cells during hyperammonia (Fig. 2J). Increased autophagic area and flux were observed in response to ammonium acetate and a combination of ammonium acetate and chloroquine (Fig. 2J). Consistent with the electron microscopy data, immunoblots of cells treated with ammonium acetate and chloroquine also showed increased autophagic flux (Fig. 2, K and L). These studies demonstrate that autophagy is increased in response to hyperammonemia in muscle cells.

An *in vivo* autophagometer in mice measures autphagic flux in response to hyperammonemia. Typically, *in vivo* autophagic studies are limited to static measurements of autophagy markers, which do not clearly distinguish between the accumulation of an autophagy marker because of an enhancement of autophagy activation or blockade of autophagic flux. Using a novel method for evaluating autophagic flux in animal models designed by Ju et al. (the “autophagometer”) (26), we examined whether hyperammonemia in mice activates autophagy in the skeletal muscle. In these studies, hyperammonemia was induced in mice by treatment with ammonium acetate intraperitoneally and autophagic flux determined by blocking lysosomal fusion with the autophagosome, using colchicine to inhibit degradation of autophagy proteins. Surprisingly, we observed only minimal LC3 lipidation in response to ammonium acetate alone. A potential explanation for this is that ammonium acetate increased autophagic flux, with a rapid degradation of LC3-II. To study autophagic flux, mice were treated with ammonium acetate for 7 days, followed by a 2-day treatment with intraperitoneal colchicine to block autophagy to measure the flux. Consistently, mice treated with ammonium acetate and colchicine showed a significant (P = 0.005) accumulation of LC3-II (Fig. 3, A–C). These studies demonstrated that hyperammonemia significantly increases autophagic flux in vivo.

**Increased skeletal muscle autophagy in hyperammonemia is not transcriptionally regulated.** To determine whether the increased autophagy during hyperammonemia is transcriptionally regulated, we carried out real-time PCR on critical genes that regulate different stages of autophagy, including ULK1, GABARAP, Atg5, Atg7, Atg4b, beclin-1, mVps34, and LAMP2. We observed a significant increase in the expression of beclin-1, Atg5, and Atg7 after 24 h of ammonium acetate treatment (Fig. 4). Similar changes were seen in the expression of ULK1, GABARAP, Atg4b, mVps34, and LAMP2 (data not shown). Because the transcriptional upregulation of these genes occurs after autophagy is induced by ammonium acetate, these data suggest that transcription of these genes is not required for initiation of autophagy, but rather, transcription is stimulated to replenish autophagy gene products that are depleted with increased autophagy in response to hyperammonemia.
Nitrated protein expression is increased in cirrhosis and colocalized to the autophagosomes. In astrocytes, hyperammonemia has been reported to induce nitration of proteins with resultant structural and functional alterations (3, 39, 41), and removal of these modified proteins may require increased autophagic degradation. To determine the mechanism by which hyperammonemia induces autophagy, we quantified tyrosine nitration of proteins using immunoblots in the skeletal muscle from human cirrhosis and the PCA rat model as well as in the C2C12 myotubes exposed to hyperammonemia. We found increased levels of nitrated proteins by immunoblots in 1) the muscle from human cirrhosis compared with controls
Cirrhosis is a state of accelerated starvation and is associated with a number of metabolic perturbations, including increased blood ammonia concentration (47). The present studies provide a potential mechanistic link involving autophagy between two...
features of cirrhosis, hyperammonemia and sarcopenia. Autophagy not only serves to degrade dysfunctional proteins and damaged organelles but also provides essential nutrients during states of starvation (21). We show an increased skeletal muscle autophagy in cirrhosis using three different measurements (LC3 lipidation, beclin-1 expression, and p62 degradation). Similar observations were made in the skeletal muscle of the PCA rat, a model that has been used to examine the mechanisms of sarcopenia in cirrhosis with portosystemic shunting. A consistent biochemical abnormality in both of these systems is hyperammonemia, and studies using in vivo mice injected with both ammonium acetate and differentiated murine C2C12 myotubes demonstrated that hyperammonemia induces skeletal muscle autophagy.

Previously, we have reported that the ubiquitin-mediated proteolysis in the PCA rat was unaltered, and this was accompanied by impaired muscle protein synthesis (10). Consistently, in the present study, we show that in patients with cirrhosis expression of the ubiquitin-proteasome components was unaltered in the skeletal muscle. Our in vitro model of murine myotubes exposed to ammonium acetate also showed that the proteasome activity was not altered, whereas markers of autophagy were increased. Similarly, markers of autophagy were increased in the skeletal muscle of the PCA rat model of sarcopenia of cirrhosis with portosystemic shunting. However, studies on static models of autophagy have been identified to have significant limitations (26, 43). Therefore, we used an in vivo autophagometer mouse model to demonstrate that hyperammonemia increases autophagic flux in vivo. Autophagic flux was determined by blocking lysosomal fusion with the autophagosome, using colchicine to inhibit the degradation of the autophagy regulatory proteins. In these studies, we observed that hyperammonemia resulted in an about fivefold increase in autophagic flux. To place this in perspective, rapamycin increases autophagic flux about twofold, and starvation, a well-known inducer of muscle autophagy, increases autophagic flux threefold (25, 26). Our studies on autophagic flux in vivo in mice show that hyperammonemia is a potent inducer of skeletal muscle autophagy. These findings, combined with our supporting observations in differentiated C2C12 myotubes and our in vitro data, are consistent with our in vivo observations in cirrhotic patients and the PCA rat model. Thus, our studies suggest that the liver-muscle axis of disease in cirrhosis is mediated through hyperammonemia and stimulation of autophagy.

In cirrhosis, impaired hepatic disposal of ammonia results in its increased skeletal muscle uptake for disposal by nonureagenic pathways (34, 35). Ammonia increases tyrosine nitration of proteins in astrocytes, and nitration of proteins has been suggested to increase autophagy (20, 33, 41, 45). Hyperammonemia may induce skeletal muscle protein nitration, with the increase in autophagy responsible for removing the nitrated proteins. Our studies showed an increased global muscle protein tyrosine nitration in response to hyperammonemia. It was interesting that in the GFP-LC3-expressing cells exposed to hyperammonemia there was a relatively rapid induction of tyrosine nitration, followed by the formation of the green autophagosome puncta and subsequent entry of the nitrated proteins into the autophagosomes. Our studies also suggest that degradation of nitrated proteins may be one of the functions of autophagy in the skeletal muscle in cirrhosis and hyperammonemia. However, this interpretation must be made cautiously since it is not clear from our observations whether the tyrosine-nitrated proteins are responsible for inducing autophagy or whether the nitrated proteins are a consequence of the ammonia-induced nitration stress placed on these cells. Although others have reported that S-nitrosylation of cysteine residues in proteins induces autophagy (19), to the best of our knowledge this is the first report that hyperammonemia-induced tyrosine nitration is associated with increased skeletal muscle autophagy. Whether nitration of autophagy-specific proteins occurs and whether it alters their function is a possibility that needs to be explored in the future since nitration of proteins have been reported to impair their activity (3, 46).

Others have shown that ammonia induces autophagy in human cells as well as in mouse embryonic fibroblasts by a ULK1/2 independent mechanism (8, 16, 30). Hyperammonemia also induces nitration of proteins by inducing cell stress and may be responsible for increased autophagy in the skeletal muscle (14). Our studies showed that the effects of ammonium acetate were not due to the alteration in intracellular pH; rather, they were due directly to the ammonia on the induction of autophagy. Further studies examining the molecular signals required for hyperammonemia-induced autophagy in muscle will be useful in determining how to influence this process clinically.

Few studies have examined the transcriptional response of autophagy components (21, 27). Our systematic studies on the expression of mRNA of the autophagy genes showed that the initial increase in autophagy in response to hyperammonemia
was not transcriptional. These observations are novel and appear to be unique to hyperammonemia-induced autophagy in muscle cells. Importantly, our observations suggest that the initial increase in autophagy is due to activation of the preexisting autophagy proteins. Delayed transcriptional upregulation of specific autophagy genes potentially occurs to replenish the proteins cleared by autophagy. Currently, the mechanism(s) responsible for the transcriptional upregulation of these genes during hyperammonemia are not known and are beyond the scope of the present studies.

Our observations show for the first time that skeletal muscle autophagy in cirrhosis is increased and suggest that the liver—muscle axis in cirrhosis is mediated by hyperammonemia. These observations suggest that studies on understanding the mechanisms by which hyperammonemia induces skeletal muscle autophagy will be of significant clinical relevance. Since ammonia lowering therapy is used in clinical practice (14, 31, 37), understanding autophagy in this context may provide novel strategies to reverse sarcopenia of cirrhosis.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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