Aging Accentuates Alcohol-Induced Decrease in Protein Synthesis in Gastrocnemius

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ABSTRACT

The present study sought to determine whether the protein catabolic response in skeletal muscle produced by chronic alcohol feeding was exaggerated in aged rats. Adult (3 month) and aged (18 month) female F344 rats were fed a nutritionally complete liquid diet containing alcohol (36% of total calories) or an isocaloric isonitrogenous control diet for 20 weeks. Muscle (gastrocnemius) protein synthesis as well as mTOR and proteasome activity did not differ between control-fed adult and aged rats, despite the increased TNFα and IL-6 mRNA and decreased IGF-I mRNA in muscle of aged rats. Compared to alcohol-fed adult rats, aged rats demonstrated an exaggerated alcohol-induced reduction in lean body mass and protein synthesis (both sarcoplasmic and myofibrillar) in gastrocnemius. Alcohol-fed aged rats had enhanced dephosphorylation of 4E-BP1 as well as enhanced binding of raptor with both mTOR and Deptor, and a decreased binding of raptor with 4E-BP1. Alcohol feeding of both adult and aged rats reduced RagA binding to raptor. The LKB1-AMPK-REDD1 pathway was up regulated in gastrocnemius from alcohol-fed aged rats. These exaggerated alcohol-induced effects in aged rats were associated with a greater decrease in muscle but not circulating IGF-I, but no further increase in inflammatory mediators. In contrast, alcohol did not exaggerate the age-induced increase in atrogin-1 and MuRF1 mRNA or the increased proteasome activity. Our results demonstrate that, compared adult rats, the gastrocnemius from aged rats is more sensitive to the catabolic effects of alcohol on protein synthesis, but not protein degradation, and this exaggerated response may be AMPK-dependent.

Key words: mTOR, protein degradation, sarcopenia, atrophy, AMPK
INTRODUCTION

Chronic alcohol abuse produces a diverse array of hormonal and metabolic derangements which alter body composition (51). One hallmark of excessive sustained alcohol consumption, evident in both humans and rodent models, is the reduction in lean body mass (LBM) and muscle mass (20, 56). The etiology of this wasting is due predominantly to a decrease in protein synthesis (48, 52, 53, 56, 67), mediated via an impairment in the kinase activity of mTOR (mammalian target of rapamycin) (50, 55), while alcohol-induced changes in muscle protein degradation are more controversial (73, 84). It is now recognized that mTOR resides in two distinct complexes (mTORC1 and mTORC2) and that alterations in protein-protein interactions within primarily mTORC1 directly regulate the rate of protein translation (23). For example, chronic alcohol consumption in rats and exposure of cultured myocytes to alcohol increase the binding of raptor (regulatory associated protein of mTOR) to mTOR (39, 55) which is consistent with the formation of a “closed confirmation” rendering mTOR less active (45). Other catabolic insults alter different protein-protein interactions within mTORC1 (14, 44, 65), but this level of granularity has not yet been investigated in response to chronic alcohol consumption.

The fraction of the US population > 65 years of age is rapidly growing. While chronic alcohol abuse has long been considered to promote premature aging, most data supporting this hypothesis have focused on the peripheral and central nervous system (3, 34, 77), with little research on whether aging alters the hormonal and metabolic response to alcohol (21). However, indirect evidence supports the possibility that alterations in protein homeostasis produced by excessive alcohol and as part of the aging process (i.e., sarcopenia) may interact to produce either an additive or synergistic effect on skeletal muscle mass. For example, aged rats are highly sensitive to the catabolic effect of the synthetic glucocorticoid Dexamethasone demonstrating a more pronounced muscle wasting than adult animals (12, 13) and the regenerative capacity of muscle is markedly diminished by alcohol feeding in aged rats (60).
Therefore, to address these gaps in understanding, we designed experiments to test the hypothesis that the catabolic effects of alcohol on skeletal muscle protein balance are exaggerated in aged rats. While the primary endpoints pertaining to protein homeostasis were related to protein synthesis, we also assess the impact of alcohol and aging on proteasome activity well as circulating and tissue factors recognized to modulated muscle protein balance.

MATERIALS AND METHODS

Animal protocol. Specific pathogen free adult (3 month) and aged (18 month) female Fischer 344 (F344) rats were purchased from the National Institute on Aging colony at Taconic (Hudson, NY). Rats were housed under a 12:12-h light-dark cycle and initially received standard rat chow (LabDiet 5001; PMI Nutrition International, St. Louis, MO) and water ad libitum for at least 1 wk before experiments were performed. Thereafter, rats were randomized to a alcohol- or control-fed group as we have previously described (79). Each group was maintained for 20 wks on the Lieber-DeCarli liquid diet (Bio-Serv, Frechtown, NJ) (58). Female rats consuming the ethanol-containing diet initially received 12% of total calories from ethanol and this percentage was gradually increased each week by 12% so all animals consumed a maximum of 36% of caloric intake from alcohol. Time-matched pair-fed control animals received a liquid diet in which isocaloric maltose-dextran was substituted for ethanol. While total caloric intake was experimentally controlled, no attempt was made to match the pattern of food intake between control and alcohol-fed rats. Consumption of the liquid diet was assessed daily and animals were weighed weekly. This duration of alcohol feeding has been previously reported to lead to muscle wasting and alterations in muscle protein balance in both male and female rats (52, 53, 55, 56). All experiments described herein were approved by the Institutional Animal Care and Use Committee of the Pennsylvania State University and adhered to the National Institutes of health (NIH) guidelines for the use of experimental animals.
**Whole-body composition and muscle protein synthesis.** The liquid diet was removed from cages at ≈ 0500 hours, at which time there was liquid remaining in all feeding tubes. Body composition was then determined non-invasively in conscious rats between 0600-0700 hours using a \(^{1}\text{H}-\text{NMR}\) analyzer (Bruker LF90 proton-NMR Minispec; Bruker Optics, Woodlands, TX), as previously described (49). In vivo protein synthesis in gastrocnemius and soleus was determined between 0800-1000 hours (i.e., ≈ 3-5 hrs after removal of food) using the flooding-dose technique (85). The order of adult and aged control- and alcohol-fed rats was randomized to minimize potential changes which might result from differences in duration of fasting from the beginning to end of the protocol. Rats were anesthetized using intraperitoneal pentobarbital (100 mg/kg) and a catheter inserted in the carotid artery. Arterial blood was collected for measuring the plasma concentration of ethanol and various hormones, and then a bolus injection of L-[2,3,4,5,6-\(^{3}\text{H}\)]phenylalanine (Phe; 150 mM, 30 \(\mu\text{Ci/ml}\); 1 ml/100 g body weight [BW]) was injected via the jugular vein. Serial arterial blood samples were drawn at 2, 6 and 10 min after Phe injection for measurement of Phe concentration and radioactivity. Immediately after the final blood sample, skeletal muscles were excised and a portion frozen between liquid-nitrogen cooled aluminum blocks and the remaining fresh muscle directly homogenized. Blood was centrifuged and plasma was collected. All tissue and plasma samples were stored at −80 °C until analyzed. The frozen muscle was powdered under liquid nitrogen and a portion used to estimate the global rate of incorporation of \[^{3}\text{H}]\text{Phe}\) into protein, whereas another portion of the frozen, powdered muscle was used to separate the myofibrillar and sarcoplasmic proteins, as described by our laboratory (85).

**Western blot analysis.** Fresh tissue was homogenized in ice-cold homogenization buffer consisting of (in mmol/L) 20 HEPES (pH 7.4), 2 EGTA, 50 sodium fluoride, 100 potassium chloride, 0.2 EDTA, 50 \(\beta\)-glycerol phosphate, 1 DTT, 0.1 phenylmethane- sulphonylfluoride, 1 benzamidine, and 0.5 sodium vanadate using a Polytron homogenizer and clarified by
centrifugation (49, 50, 52, 53, 55). Equal amounts of protein per sample were subjected to standard SDS-PAGE for total and phosphorylated ribosomal protein S6 kinase-1 (S6K1; Thr389; Cell Signaling, Beverly, MA) and eukaryotic initiation factor 4E binding protein-1 (4E-BP1; Thr 37/46; Bethyl Laboratories, Montgomery, TX). In addition, total and phosphorylated (Thr246) PRAS40, total and phosphorylated (Ser792) raptor, total and phosphorylated (Thr172) 5'-AMP-activated kinase (AMPKα), as well as total GβL (also called mLST8), DEPTOR, RagA and RagC, and REDD1 (regulated in development and DNA damage responses) were also determined by Western blot analysis. Blots were developed with enhanced chemiluminescence Western blotting reagents and then exposed to X-ray film in a cassette equipped with a DuPont Lightning Plus intensifying screen. After development, the film was scanned (Microtek ScanMaker IV) and analyzed using NIH Image 1.6 software.

Muscle was also homogenized in CHAPS (3[(3-cholamidopropyl)dimethylammonio]-propanesulfonic acid) buffer consisting of (in mmol/L) 40 HEPES, pH 7.5, 120 NaCl, 1 EDTA, 10 pyrophosphate, 10 β-glycerol phosphate, 50 sodium fluoride, 1.5 sodium vanadate, 0.3% CHAPS, and 1 protease inhibitor cocktail tablet (44). The homogenate was mixed on a platform rocker and clarified by centrifugation. An aliquot of the resulting supernatant was combined with anti-raptor antibody and immune complexes isolated with a goat anti-rabbit BioMag IgG beads (PerSeptive Diagnostics, Cambridge, MA). Beads were collected, washed with CHAPS buffer, precipitated by centrifugation, and subjected to SDS-PAGE and analysis as above.

**RNA extraction and real-time quantitative PCR.** Total RNA was extracted using Tri-reagent (Molecular Research Center, Inc., Cincinnati, OH) and RNeasy mini kit (Qiagen, Valencia, CA) following manufacturers’ protocols. Gastrocnemius was homogenized in tri-reagent followed by chloroform extraction according to the manufacturer’s instruction. An equal volume of 70% ethanol was added to the aqueous phase and the mixture was loaded on a Qiagen mini spin column. The Qiagen mini kit protocol was followed from this step onwards
including the on-column DNase I treatment to remove residual DNA contamination. RNA was eluted from the column with RNase-free water and an aliquot was used for quantitation (NanoDrop 2000, Thermo Fisher Scientific, Waltham, MA). Quality of the RNA was analyzed on a 1% agarose gel. Total RNA (1 μg) was reversed transcribed using superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) following manufacturer's instruction. Real-time quantitative PCR was performed using 25 ng of cDNA in a StepOnePlus system using TaqMan gene expression assays (Applied Biosystems, Foster City, CA) for: Atrogin-1 (F-box protein 32; Rn00591730_m1), muscle RING-finger 1 (MuRF1; Rn00590197_m1), interleukin (IL)-6 (Rn01410330_m1), IL-1 (Rn00566700_m1), tumor necrosis factor (TNF)-α (Rn01525859_g1), nitric oxide synthase (NOS)-2 (Rn00561646_m1), insulin-like growth factor-I (IGF-I; Rn00710306_n1 - all IGF-I transcripts), IGF binding protein (IGFBP)-4 (Rn01464112_m1) and IGFBP-5 (Rn00563116_m1), L32 (Rn00820748_g1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Rn01775763 g1). The comparative quantitation method 2-ΔΔCt was used in presenting gene expression of target genes in reference to the endogenous control.

Proteasome activity. Fresh gastrocnemius was homogenized in cell lysis buffer containing (in mM) 25 HEPES, 5 MgCl₂, 5 EDTA, 5 DTT, pH 7.5 followed by centrifugation at 14,000 rpm (55). The protein concentration in the supernatant was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL). The proteasome enzymatic activity was measured by using a proteasome 20S assay kit (Enzo Life Sciences, Farmingdale, New York) following the manufacturer's instructions. Briefly, the protein extract from gastrocnemius was used to assess proteasome 20S activity by measuring the hydrolysis of a fluorogenic peptidyl substrate Suc-Leu-Leu-Val-Tyr-AMC (AMC: 7-amino-4-methylcoumarin). This substrate was cleaved by the proteasome activity and the subsequently released free AMC was then detected by a fluorometer (excitation wavelength 380 nm; emission wavelength 460 nm). The fluorescence signal was monitored before and 1 h after incubation at 37 °C. The change in fluorescence
signal was normalized to protein content. Each sample/substrate combination was measured both in the presence and in the absence of the specific 20S proteasome inhibitor MG132 (Boston Biochem, Cambridge, MA) to account for any nonproteasomal degradation of the substrate, as previously described (57).

**Plasma concentrations.** Plasma insulin (Alpco; Salem, NH) and IGF-I (R & D Systems, Minneapolis, MN) concentrations were measured using commercial ELISAs. The plasma glucose and alcohol concentrations were determined by a rapid analyzer (Analox Instruments, Lunenburg, MA). Levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined by standard enzymatic procedures (Sigma-Aldrich; St. Louis, MO). Finally, the plasma branched-chain amino acid concentrations were determined using reverse-phase HPLC after precolumn derivatization of amino acids with phenylisothiocyanate. The plasma concentrations of glucose, insulin, IGF-I, branched-chain amino acids and alcohol were determined on blood collected immediately prior to injection of radiolabeled phenylalanine.

**Statistical analysis.** Data for each condition are summarized as means ± standard error of the mean (SEM) where the number of rats per group is indicated in the figure or table legend. Statistical evaluation of the data was performed using ANOVA followed post-hoc by Student-Neuman-Keuls test. Differences were considered significant when $P < 0.05$.

**RESULTS**

**Body composition.** The body weight of adult rats fed the alcohol-containing diet tended to be reduced (11%), compared to pair-fed control rats, but this change did not achieve statistical significance (Figure 1A). The body weight of both groups of aged rats was increased, compared to control-fed adult rats, but the body weight of pair-fed alcohol consuming aged rats was reduced 17%. Alcohol consumption in adult rats significantly decreased the percent LBM and tended to increase the percent body fat, compared to control-fed rats of the same age.
(Figure 1B and 1C, respectively). The percentage of LBM was reduced and the percentage of fat mass was increased in alcohol-fed aged rats, compared to both adult groups. The qualitative changes in the mass of the gastrocnemius, a representative fast-twitch muscle, were comparable to those observed for LBM (data not shown). However, because of the difference in body weight between adult and aged rats, we also normalized the gastrocnemius weight to total body weight. In doing so, the relative gastrocnemius weight was lower in aged rats than adult animals and, although not statistically significant, there was a trend for alcohol-fed rats in both groups to have a lower relative gastrocnemius weight than the age-matched pair-fed control rats (Figure 1D).

**Skeletal muscle protein synthesis and mTOR activity.** The rate of total protein synthesis in gastrocnemius did not differ between control-fed adult and aged rats (Figure 2). There was also no difference in the synthetic rate for sarcoplasmic or myofibrillar proteins between these groups. In contrast, alcohol feeding reduced total, sarcoplasmic and myofibrillar protein synthesis by 15-20% in adult rats. Rates of synthesis for total, sarcoplasmic and myofibrillar proteins were decreased in aged rats fed alcohol (45-55%) and the reductions in each were greater than that seen in the alcohol-fed adult rats (Figure 2).

In contrast, no significant age or alcohol-induced change in mass or protein synthesis was detected in the soleus, a predominantly slow-twitch muscle (data not shown), and therefore no subsequent analysis was performed on this muscle type.

mTOR regulates protein translation, at least in part, by phosphorylation of downstream targets S6K1 and 4E-BP1 (23). In adult rats, an alcohol-induced decrease in mTOR, 4E-BP1 and S6K1 phosphorylation was detected, independent of a change in the total amount of these individual proteins (Figure 3). A similar alcohol-induced decrease in mTOR and S6K1 phosphorylation occurred in gastrocnemius of aged rats, while the reduction in 4E-BP1 phosphorylation was greater in aged rats, compared to alcohol-fed adult rats. We failed to
detect a significant change in either the phosphorylation state or total amount of these three proteins in gastrocnemius from adult and aged rats fed the control diet.

The mTORC1 complex is composed of at least five proteins – mTOR, raptor, GβL, PRAS40 and Deptor (23). Western blot analysis of whole muscle homogenates did not demonstrate a significant age- and/or alcohol-induced change for the total amount of raptor, mTOR, PRAS40 or GβL (Figure 4A). Although the total amount of raptor did not differ between groups, Ser792-phosphorylation of raptor was increased in alcohol-fed adult and aged rats (Figure 4A and 4B). Aged, but not adult, rats had a 2-fold increase in Deptor when fed the alcohol-containing diet (Figure 4A and 4C).

The activity of mTORC1 is controlled in part by various protein-protein interactions. In this regard, raptor functions as a scaffold protein recruiting substrates to mTORC1 via short TOS (mTOR signaling) motifs in its substrates, thereby regulating kinase activity (64). Therefore, raptor was immunoprecipitated from gastrocnemius and then immunoblotted for PRAS40, 4E-BP1, Deptor and raptor. Alcohol feeding increased the binding of both mTOR and Deptor to raptor in adult rats and this increased binding was exaggerated in aged animals fed alcohol (Figure 5). Conversely, alcohol reduced the amount of the raptor•4EBP1 complex in gastrocnemius from aged rats to a greater extent than in adult animals. The interaction of mTOR and 4E-BP1 with raptor did not differ in control-fed adult and aged rats, but there as an increased assembly of the Deptor•raptor complex in aged rats.

Finally, the Rag GTPase proteins (i.e., RagA-D) are amino acid-specific regulators of mTORC1 which interact directly with raptor (76). Western blotting of whole tissue homogenate indicated the total amount of RagA and RagC did not differ with alcohol or aging (Figure 6, top panel). In contrast, the amount of RagA•raptor complex was reduced >90% by alcohol feeding, regardless of the age of the rat (Figure 6, bottom panel).

*Upstream protein factors regulating protein synthesis.* The general energy state of muscle is sensed by AMPK, which is activated (phosphorylated) by various catabolic agents
thereby inhibiting mTOR kinase activity (33). AMPK is an LKB1 substrate and increased LKB1 Ser428 phosphorylation is important in activating AMPK. Moreover, REDD1 is a novel stress-response gene activated in an AMPK-dependent manner (16). The total and phosphorylation stated of AMPK and LKB1 and total REDD1 did not differ in gastrocnemius from alcohol-fed adult rats nor in control aged rats, compared to adult control values (Figure 7). However, there was a 50% increase in LKB1 phosphorylation, a 3-fold increase in AMPK phosphorylation and a nearly 5-fold increase in REDD1 protein in gastrocnemius of aged rats consuming alcohol, demonstrating upregulation of the LKB-AMPK-REDD1 pathway.

**Protein degradation.** Because in vivo determinations of muscle protein breakdown are not routinely performed, we assessed accepted surrogate markers of proteolysis (5, 78). Alcohol consumption in adult animals increased the mRNA content for both atrogin-1 and MuRF1 in gastrocnemius (Figure 8A and 8B). A comparable increase in both atrogene was observed in control-fed aged rats, compared to adult control animals. However, atrogin-1 and MuRF1 mRNA content was similarly elevated in both control and alcohol-fed aged rats. Finally, in vitro proteasome activity in gastrocnemius was not altered by alcohol feeding in adult rats, but was increased 25% in control-fed aged rats (Figure 8C). The magnitude of the age-induced increase in proteasome activity did not differ between control- and alcohol-fed aged rats.

**IGF system.** Alterations in circulating and local concentrations of IGF-I and its various binding proteins can impact muscle protein metabolism (22). Alcohol feeding in adult rats decreased both the plasma concentration and muscle mRNA content for IGF-I by approximately 30% (Figure 9A and 9B). A similar reduction in blood and muscle IGF-I was observed in control-fed aged rats. However, whereas alcohol feeding did not further reduce the plasma IGF-I concentration in aged rats, this group exhibited the lowest IGF-I mRNA content in gastrocnemius. IGFBP-4 and IGFBP-5 can regulate IGF-I bioavailability and changes in these binding proteins have been noted in some types of muscle disease (54). We detected no alcohol- or age-related change in IGFBP-4 mRNA in gastrocnemius (Figure 9C). In contrast,
IGFBP-5 mRNA was increased 50% in both groups of aged rats compared to adult animals, but muscle IGFBP-5 was not altered by alcohol feeding (Figure 9D).

**Cytokines and inflammatory mediators.** Cytokines and inflammatory mediators have been implicated in the etiology of muscle wasting (24). Alcohol increased TNFα mRNA content 3-fold in the gastrocnemius from adult animals (Figure 10A). Muscle TNFα mRNA was increased 2-fold in control-fed aged rats, but alcohol-feeding did not lead to a further increase. IL-6 mRNA content was also increased in alcohol-fed adult animals (Figure 10B). The gastrocnemius from both control- and alcohol-fed aged rats showed a 4-fold increase in IL-6 mRNA. In contrast, we detected no alcohol- or age-induced change in either IL-1β or NOS2 mRNA in gastrocnemius (Figure 10C and 10D).

**Metabolic substrates and hormones.** The total amount of alcohol consumed by aged rats tended to be greater than adult rats (3.7 ± 0.3 vs 3.0 ± 0.2 g ethanol/rat/day, respectively; \( P = 0.07 \)); however, when normalized per kg of body weight, alcohol consumption was lower in aged compared to adult rats (10.6 ± 0.9 vs 13.1 ± 0.7 g ethanol/kg/day, respectively; \( P < 0.05 \)). The blood alcohol concentration (BAC) determined near the beginning of the light cycle did not differ between adult and aged rats, averaging \( \approx 16 \) mM (\( \approx 70 \) mg/dL; 0.07%; Table 1). There were no alcohol- or age- induced changes in the concentration of insulin, a known anabolic hormone. The constant insulin levels coupled with the lack of alcohol- or age-induced change in plasma glucose, suggests the absence of overt insulin resistance. Finally, the plasma concentration of each branched-chain amino acid (leucine, isoleucine and valine) did not differ in alcohol-fed or aged rats, compared to control values. Finally, ALT and AST were determined to estimate hepatic damage. As presented in Table 1, ALT and AST did not differ between control-fed adult and aged rats, but both liver enzymes were increased to a comparable extent by alcohol feeding.

**DISCUSSION**
The current data support our hypothesis that the catabolic effect of alcohol on gastrocnemius is exaggerated in aged rats and this response appears largely mediated by mTOR-dependent changes in protein synthesis. To place these new data in the context of existing literature, we will first discuss pertinent and novel changes observed in response to alcohol and aging alone before concluding with a discussion of the age-alcohol interaction on protein balance.

**Alcohol-induced changes in adult female rats.** The current data are consistent with previous reports indicating that alcohol decreases both sarcoplasmic and myofibrillar protein synthesis without an apparent increase in protein degradation (71, 83). The alcohol-induced decrease in gastrocnemius protein synthesis is caused at least in part by a decreased mTOR kinase activity, as evidenced by the decreased autophosphorylation of mTOR as well as decreased phosphorylation of the downstream substrates 4E-BP1 and S6K1 (50, 52, 55, 56). While the activity of mTORC1 is regulated by various protein-protein interactions, changes have only been reported under in vitro conditions with relatively short-term incubation of myocytes with alcohol (37, 39, 40). While there was no difference in the total amount of raptor, Deptor, PRAS40, GβL or RagA/C in homogenates of gastrocnemius from control- and alcohol-fed adult rats, alcohol-induced changes were detected for several potentially important protein-protein interactions within mTORC1. For example, alcohol-fed rats have an increased binding of Deptor to raptor with a concomitant reduction in the binding of 4E-BP1 with raptor. This alcohol-induced increase in Deptor•raptor complex formation is particularly noteworthy as Deptor is a negative regulator of mTORC1 activity (69). Previous studies have reported that overexpression of Deptor increases cell proliferation while Deptor knock down can reverse the muscle atrophy produced by disuse (43). Moreover, increased Deptor•raptor binding occurs in myocytes cultured with alcohol (39). The alcohol-induced reduction in raptor•4EBP1 binding is consistent with the scaffold function of raptor and the necessary recruitment of 4E-BP1 prior to its phosphorylation and release (36). Moreover, the increased raptor S792-phosphorylation in
alcohol-fed rats is consistent with inhibition of mTOR kinase in other catabolic conditions (29). Whereas the increased raptor phosphorylation is typically attributed to activation of the cellular energy sensor AMPK (33), this pathway does not appear activated because the phosphorylation of AMPK and its upstream kinase LKB1 and a downstream substrate REDD1 did not differ between adult control- and alcohol-fed rats. We also detected a reduced binding of RagA with raptor in response to alcohol, a situation which would be expected to impair protein synthesis (76), and which has only been previously reported in myocytes incubated short-term with alcohol (38).

Relatively less is known regarding alcohol-induced changes in muscle proteolysis. We have reported that while acute alcohol intoxication increases the mRNA content for the ubiquitin-E3 ligases MuRF1 and atrogin-1, there was no evidence of increased proteolysis based on either 3-methylhistidine release from isolated perfused muscles or tyrosine release from incubated epitrochlearis muscles (84). Increased MuRF1 and atrogin-1 have also been observed in muscle from alcohol-fed rats (53, 66). Although our current results confirm these earlier observations, proteasome activity did not differ between control and alcohol-fed rats. These data would suggest that if chronic alcohol intake increases proteolysis in gastrocnemius, the mechanism must be mediated by other pathways for protein breakdown or that the degradation of a relatively small number of specific proteins is enhanced (90).

Excess production of proinflammatory cytokines can decrease muscle protein synthesis (24), and an increase in muscle TNFα and IL-6 mRNA is observed in some (8, 66) but not other studies (62) in response to chronic alcohol feeding. Our current data confirm alcohol-fed rats have a selective increase in TNFα and IL-6 mRNA in gastrocnemius, but no detectable change in IL-1β or NOS2. The lack of alcohol-induced change in NOS2 was unexpected as this inflammatory mediator has potent inhibitory effects on muscle protein synthesis (25). Finally, alcohol consumption decreases both circulating and muscle IGF-I (52, 55, 75) which may represent a potential mechanism for the decreased muscle protein synthesis (18, 22). Our
results confirm that alcohol-fed rats have both a reduction in plasma IGF-I and muscle IGF-I mRNA, but no change in either IGFBP-4 or IGFBP-5 which can modulate IGF-I bioavailability (22). We speculate that collectively the features observed in adult alcohol-fed mice (i.e., atrophy, decreased protein synthesis, decreased circulating and tissue IGF-I and increased atrogenes and specific inflammatory cytokines) are consistent with a premature aging-like phenotype (described below). Other characteristics of this phenotype, such as fibrosis, lipid accumulation and impaired regeneration, will need to be assessed in future studies.

**Age-induced changes.** Whether an absolute age-related decrease in muscle mass is detected in rats appears dependent on a complex interaction of multiple factors, including animal husbandry, rat strain, muscle type, sex, type of food provided, presence of comorbid conditions, and a broad definition of what age constitutes “old” in rats (35, 41, 91). However, essentially all studies report the presence of sarcopenia when muscle mass is normalized to body weight, and such was the case in the current study. The confounders mentioned above may have also influenced other metabolic and hormonal endpoints related to muscle protein balance, thereby contributing to the discrepancy on age-induced changes in the literature. For example, although some studies have shown aging decreases (4, 70, 88) or even increases mixed or myofibrillar muscle protein synthesis (47, 80), other studies find the synthetic rates for mitochondrial, sarcoplasmic and myosin heavy chain fractions in muscle are well maintained during aging (11, 27, 81, 82). Our data support these latter reports and reveal no difference in the synthetic rate for total, myofibrillar or sarcoplasmic proteins in gastrocnemius. The lack of an age-induced change in muscle protein synthesis is consistent with the similar content of total and phosphorylated proteins controlling mRNA translation (e.g., mTOR, 4E-BP1, S6K1, raptor, Deptor, RagA/C). Moreover, there was no change in the phosphorylation state of either AMPK or LKB1 or total REDD1, similar to previous studies (74), suggesting that aging did not alter constitutive AMPK activity in gastrocnemius.
Age-induced changes in atrogen expression and their physiological importance, if any, in regulating muscle proteolysis remains unclear. For example, several studies have reported no age-induced change in MuRF1 and atrogin-1 (7, 26, 89), while others showed an increased (1, 9, 10) or decreased (15, 18) expression of these atrogens. We observed a 2- to 3-fold increase in both atrogin-1 and MuRF1 mRNA in aged rats which was associated with a concomitant stimulation of in vitro-determined proteasome activity. These data are consistent with the elevated rate of proteolysis observed in aged humans (87) and elevated rate of 3-methylhistidine excretion in rats (63). Although these data suggest sarcopenia may be due solely to an increase in proteolysis, as opposed to a decrease in protein synthesis, the assessment of protein metabolism was conducted in the postabsorptive state (≈3-5 h after removal of food) and aging has been shown to attenuate the anabolic response of muscle protein synthesis to nutrient stimulation (2, 6). Hence, the relative importance of changes in protein synthesis and degradation as a cause for sarcopenia may vary depending on nutritional fluctuations.

Circulating IGF-I was reduced in control-fed aged rats, consistent with previous observations (17, 86). Locally produced IGF-I is also implicated in maintenance of muscle mass. However, data on IGF-I mRNA in skeletal muscle per se is often contradictory, with our study and others (9, 59, 86) showing a decrease, but others reporting either no change or an increased muscle IGF-I with aging (11, 18, 30, 32). Our current data do little to reconcile these highly diverse findings. Finally, aging can increase the circulating concentration of a number of potentially catabolic inflammatory mediators (68, 82) as well the TNFα mRNA content in muscle (9). In this regard, both TNFα and IL-6 mRNA were increased in gastrocnemius from aged rats.

**Alcohol-aging interaction.** The primary focus of the current study was to elucidate the combined effect of chronic alcohol consumption and aging on muscle protein balance. Our results highlight that aged rats have an increased sensitivity to the catabolic effects of dietary alcohol. A comparable increased sensitivity to excess glucocorticoids has been reported in aged
rats (13). Sustained alcohol consumption in aged female rats produced an exaggerated decline in both LBM and the absolute mass of the gastrocnemius. Likewise, the alcohol-induced decrease in gastrocnemius protein synthesis (global, sarcoplasmic and myofibrillar) was of greater magnitude in aged rats. No such age-alcohol interaction was detected for muscle weight or protein synthesis in the soleus muscle which is consistent with the preferential effect of alcohol feeding on fast-twitch fibers (e.g., gastrocnemius) vs slow-twitch fibers (soleus) (55). The mechanism for this relatively selective atrophy in response to alcohol is not known.

This exaggerated drop in muscle protein synthesis was associated with a concomitant reduction in the extent of phosphorylated 4E-BP1, but not mTOR or S6K1 phosphorylation. Of the proteins which constitute the mTORC1 complex, only the negative-regulatory protein Deptor was increased by the combination of alcohol and aging. As a result of these changes, we detected an increased formation of the Deptor•raptor complex, with a reduction in the 4E-BP1•raptor complex. We speculate these changes in mTORC1 are causally related to the accentuated decrease in muscle protein synthesis in the alcohol-fed aged rats (38, 43, 44). These changes in protein-protein interaction within mTORC1 may be LKB1-AMPK-REDD1-dependent as this signaling pathway was only activated in muscle from alcohol-fed aged rats. However, AMPK inhibition of mTOR activity and protein synthesis is typically mediated by increased phosphorylation of raptor (30, 72), which showed no additive effect. It is noteworthy that it was the combination of alcohol and aging which stimulated the AMPK pathway, an activation which was not seen with either factor alone.

The physiological mechanism for this enhanced catabolic response remains to be elucidated. Of the parameters assessed, we detected no interaction of alcohol and aging on the plasma concentrations of IGF-I, insulin or branched-chain amino acids as well as no additional increased in TNFα or IL-6 within gastrocnemius. In contrast, we detected a further reduction in IGF-I mRNA content in muscle of alcohol-fed aged rats suggesting a possible mechanism.
In contrast to protein synthesis, aged rats feed alcohol had no further increase in atrogate expression or proteasome activity in gastrocnemius, compared to alcohol-fed adult rats. While our data suggest the increased sensitivity of older rats toward the catabolic effect of alcohol is predominantly mediated by a change in protein synthesis, we cannot exclude the possibility that the combination of aging and alcohol enhances other pathways of protein degradation which were not part of this investigation. In this regard, acute alcohol intoxication has been reported to increase autophagy in cardiac muscle via an AMPK-dependent mechanism (28).

Based on our data we cannot reach a definitive conclusion as to whether the increased sensitivity observed in alcohol-fed aged rats is mediated by a difference in the BAC. While alcohol consumption per kg body weight was 20% lower in aged rats and there was no difference in the BAC between groups determined at the beginning of the light cycle, we cannot exclude the possibility that aged rats had higher BAC during the dark cycle when the majority of alcohol is consumed. Such an age-related increase in BAC might be predicted if alcohol clearance is decreased by aging. However, results from pharmacokinetic studies on ethanol metabolism in rats are conflicting (31, 46), thereby rendering any conclusion equivocal. We can also not exclude the possibility that an alcohol- and/or aging-induced decrease in spontaneous physical activity did not contribute in part to the observed muscle phenotype. Finally, although a similar degree of hepatic injury in alcohol-fed adult and aged rats is suggested by the similar increment in AST and ALT levels, we have previously reported exaggerated hepatic lipidosis and inflammation in alcohol-fed aged rats (79). Therefore, detailed studies which assess hepatic function directly are needed to determine whether metabolic disturbances secondary to hepatic dysfunction (e.g., hyperammonemia) are causally related to the muscle wasting detected in alcohol-fed aged rats.

Our results demonstrate that chronic alcohol consumption has a greater catabolic effect on muscle wasting in aged versus adult female rats. The mechanism for this exaggerated
response in gastrocnemius is likely mediated by changes in protein-protein interactions within mTORC1 via an AMPK-dependent mechanism.

The potential clinical relevance of in vivo-determined data is dependent in large part on the fidelity of the preclinical animal model used. In this regard, while there is no single best model, our study used the F344 rat strain because it is one of the best characterized rodent models of gerontological research (91). Additionally, the Lieber-DeCarli alcohol-containing diet has been extensively used for almost 50 years (58). Rats provided this liquid diet consume a relatively large quantity of alcohol, compared to humans classified as heavy drinkers (2 drinks/day). For example, the typical caloric intake for men is approximately 2000 kcal/day. Therefore, in humans where alcohol constitutes 36% of total calories of dietary intake (i.e., 720 kcal ethanol/day), approximately 100 g of ethanol (7 Kcal/g ethanol) would be ingested daily. As a “standard drink” averages 14 g ethanol, humans would have to consume at least 7 standard drinks per day to match that ingested by the alcohol-fed rats. However, this increased intake in rats is also coupled with a several-fold greater rate of alcohol clearance, compared to humans (19, 42). As a result, chronic alcohol ingestion in both rats and humans yields comparable circulating alcohol concentrations of 100-200 mg/dl. Collectively, our results suggest that sustained excessive alcohol consumption by the elderly should be discouraged to minimize the sarcopenia typically seen in this patient population, as muscle mass and strength are predictive of disability and all-cause mortality (61),
ACKNOWLEDGMENTS

We are grateful to Gina Deiter and Anne Pruznak for excellent technical assistance.

GRANTS

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FIGURE LEGENDS

Figure 1. Effect of alcohol consumption on total body weight, body composition and gastrocnemius mass of adult and aged female rats. Whole-body fat mass and lean body mass (LBM) were determined in vivo using $^1$H-NMR and normalized to the body weight (BW) of each rat. Values are means ± SEM; N = 8-9 rats per group. Values with different letters (a,b,c) are statistically different ($P < 0.05$); values with the same letter are not statistically different.

Figure 2. Effect of alcohol consumption on in vivo-determined protein synthesis in gastrocnemius of adult and aged female rats. Values are means ± SEM; N = 8-9 rats per group. Values with different letters (a,b,c) are statistically different ($P < 0.05$); values with the same letter are not statistically different.

Figure 3. Effect of alcohol consumption on total and phosphorylated mTOR, 4E-BP1 and S6K1 in gastrocnemius of adult and aged female rats. Values are means ± SEM; N = 8-9 rats per group. Bar graphs, quantitation of all Western blot data for Ser2481-phosphorylated mTOR (autophosphorylation site), Thr37/46-phosphorylated 4E-BP1 and Thr389-phosphorylated S6K1 normalized to the total amount of the respective protein, and the control-fed value set at 100 arbitrary units (AU). C = control-fed and A = alcohol-fed. Values with different letters (a,b,c) are statistically different ($P < 0.05$); values with the same letter are not statistically different.
**Figure 4.** Effect of alcohol consumption on protein components of mTORC1 in gastrocnemius of adult and aged female rats. Values are means ± SEM; N = 8-9 rats per group. Western blot data normalized to the amount of total raptor or tubulin, with the control-fed value for adult rats set at 100 AU. C = control-fed and A = alcohol-fed. Values with different letters (a,b,c) are statistically different (P < 0.05); values with the same letter are not statistically different.

**Figure 5.** Effect of alcohol consumption on binding of various proteins to raptor in gastrocnemius of adult and aged female rats. Values are means ± SEM; N = 5-6 rats per group. Raptor was immunoprecipitated (IP) and then immunoblotted for mTOR, 4E-BP1, Deptor, PRAS40 and raptor. Quantitation of Western blot data normalized to amount of raptor in IP, with the control-fed value for adult rats set at 100 AU. C = control-fed and A = alcohol-fed. The black vertical line in the 4EBP-1 blot indicates a spliced figure which was necessitated because the order of the adult and age rats was inverted; however, all samples on this blot were run simultaneously on the same gel, just their order reversed. Values with different letters (a,b,c) are statistically different (P < 0.05); values with the same letter are not statistically different.

**Figure 6.** Effect of alcohol consumption on total RagA and RagC as well as the binding of RagA to raptor in gastrocnemius of adult and aged female rats. Top panel, Western blot for the total amount of RagA and RagC in whole muscle homogenate where mTOR was used as loading control. There were no age and/or alcohol effect on total RagA or RagC (n = 8-9 rats per group). Bottom panel, raptor was immunoprecipitated and RagA and raptor immunoblotted. Alcohol consumption, regardless of age, reduced binding of RagA to raptor (n = 4 per group).
Figure 7. Effect of alcohol consumption on total and phosphorylated AMPK and LKB1 as well as total REDD1 protein in gastrocnemius of adult and aged female rats. Values are means ± SEM; N = 8-9 rats per group. Bar graphs, quantitation of all Western blot data for Thr172-phosphorylated AMPK and Ser428-phosphorylated LKB1 or REDD1 normalized to the total amount of the respective protein or loading control (i.e., eIF4E), and the control-fed value from adult rats set at 100 AU. C = control-fed and A = alcohol-fed. Values with different letters (a,b,c) are statistically different ($P < 0.05$); values with the same letter are not statistically different.

Figure 8. Effect of alcohol consumption on surrogate markers of protein breakdown in gastrocnemius of adult and aged female rats. Values are means ± SEM; N = 8-9 rats per group. Values with different letters (a,b,c) are statistically different ($P < 0.05$); values with the same letter are not statistically different.

Figure 9. Effect of alcohol consumption on insulin-like growth factor (IGF)-I in blood and IGF-I, IGF binding protein (IGFBP)-4 and IGFBP-5 mRNA content in gastrocnemius of adult and aged female rats. mRNA values were normalized to L32. Values are means ± SEM; N = 8-9 rats per group. Values with different letters (a,b,c) are statistically different ($P < 0.05$); values with the same letter are not statistically different.

Figure 10. Effect of alcohol consumption on inflammatory mediators in gastrocnemius of adult and aged female rats. mRNA values were normalized to L32. Values are means ± SEM; N = 8-9 rats per group. Values with different letters (a,b,c) are statistically different ($P < 0.05$); values with the same letter are not statistically different.
Table 1. **Effect of alcohol consumption on plasma concentrations of various substances in adult and aged rats**

<table>
<thead>
<tr>
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<th>Adult Alcohol</th>
<th>Aged Control</th>
<th>Aged Alcohol</th>
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<tbody>
<tr>
<td>Alcohol, mmol/L</td>
<td>ND</td>
<td>15.4 ± 2.3</td>
<td>ND</td>
<td>16.1 ± 2.8</td>
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<td>Glucose, mmol/L</td>
<td>5.4 ± 0.2</td>
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<td>5.5 ± 0.3</td>
<td>5.2 ± 0.2</td>
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<td>Insulin, pmol/L</td>
<td>162 ± 12</td>
<td>153 ± 15</td>
<td>151 ± 19</td>
<td>164 ± 27</td>
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<td>Leucine, µmol/L</td>
<td>156 ± 9</td>
<td>151 ± 7</td>
<td>139 ± 18</td>
<td>147 ± 12</td>
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<tr>
<td>Isoleucine, µmol/L</td>
<td>117 ± 21</td>
<td>98 ± 13</td>
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<tr>
<td>Valine, µmol/L</td>
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<td>ALT, U/L</td>
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<td>84 ± 8&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>AST, U/L</td>
<td>94 ± 8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>145 ± 12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>103 ± 21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>164 ± 17&lt;sup&gt;b&lt;/sup&gt;</td>
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</table>

Values are means ± SEM; N = 8-9 rats per group. There were statistically significant differences among the four groups for any listed parameter. ALT – alanine aminotransferase; AST – aspartate aminotransferase (AST). ND = not detectable. Values in the same row with a different superscript letter (<sup>a,b</sup>) are statistically different (P < 0.05); values with the same letter are not different.
REFERENCES


11. Dardevet D, Sornet C, Attai x D, Baracos VE, and Grizard J. Insulin-like growth factor-1


37. **Hong-Brown LQ, Brown CR, Kazi AA, Huber DS, Pruznak AM, and Lang CH.** Alcohol and PRAS40 knockdown decrease mTOR activity and protein synthesis via AMPK signaling


61. Metter EJ, Talbot LA, Schrager M, and Conwit R. Skeletal muscle strength as a


Figure 1

A. Body Weight (g)

B. Lean Body Mass (% BW Wt)

C. Fat Mass (% BW Wt)

D. Gastrocnemius Wt (g/g BW)
Figure 2

A

Total Protein Synthesis (nmol Phe/g wet wt/h)

Control   Alcohol

Adult    Aged

B

Sarcoplasmic Protein Synthesis (nmol Phe/mg protein/h)

Adult    Aged

C

Myofibrillar Protein Synthesis (nmol Phe/mg protein/h)

Adult    Aged
Figure 3

A

mTOR phosphorylation (Ser2481 AU/total)

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B

4E-BP1 phosphorylation (Thr37/46 AU/total)

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C

S6K1 phosphorylation (Thr389 AU/total)

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D

Adult | Aged

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Figure 4

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**B**

![Bar chart](chart1)  
- Control  
- Alcohol

**C**

![Bar chart](chart2)  
- Adult  
- Aged

Legend:
- Control
- Alcohol
Figure 5

A. Control vs Alcohol binding of mTOR and raptor.

B. Binding of 4E-BP1 and raptor.

C. Binding of Deptor and raptor.

D. IP: Raptor with mTOR, 4E-BP1, Deptor, PRAS40, and Raptor.
<table>
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<td>Total mTOR</td>
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<td>Raptor</td>
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Figure 7

A. AMPKα phosphorylation (Thr172 AU/total) across Adult and Aged conditions.

B. LKB1 phosphorylation (Ser428 AU/total) across Adult and Aged conditions.

C. REDD1 expression (AU/total eIF4E) across Adult and Aged conditions.

D. Western blot analysis showing P-AMPK (T172), Total AMPK, P-LKB1 (S428), Total LKB1, REDD1, and eIF4E (loading) for Adult and Aged conditions.
Figure 8

A. Atrogin-1 mRNA (AUL/32)

B. MuRF1 mRNA (AUL/32)

C. Proteasome Activity (µmol/min/mg protein)
Figure 9
Figure 10

A) TNF-α mRNA (AU/L32)

B) IL-6 mRNA (AU/L32)

C) IL-1β mRNA (AU/L32)

D) NOS2 mRNA (AU/L32)

Comparison between Control and Alcohol groups for each condition (Adult and Aged).