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Chronic alcohol consumption disrupts myocardial protein balance and function in aged, but not adult, female F344 rats

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Lang CH, Korzick DH. Chronic alcohol consumption disrupts myocardial protein balance and function in aged, but not adult, female F344 rats. Am J Physiol Regul Integr Comp Physiol 306: R23–R33, 2014. First published November 13, 2013; doi:10.1152/ajpregu.00414.2013.—The purpose of this study was to assess whether the deleterious effect of chronic alcohol consumption differs in adult and aged female rats. To address this aim, adult (4 mo) and aged (18 mo) F344 rats were fed a nutritionally complete liquid diet containing alcohol (36% total calories) or an isocaloric isonitrogenous control diet for 20 wk. Cardiac structure and function, assessed by echocardiography, as well as myocardial protein synthesis and proteolysis did not differ in either alcohol- versus control-fed adult rats or in adult versus aged control-fed rats. In contrast, cardiac function was impaired in alcohol-fed aged rats compared with age-matched control rats. Additionally, alcohol feeding decreased cardiac protein synthesis that was associated with decreased phosphorylation of 4E-BP1 and S6K1. This reduction in mammalian target of rapamycin (mTOR) kinase activity was associated with reduced eIF3f and binding of both Raptor and eIF4G to eIF3. Proteasome activity was increased in alcohol-fed aged rats with a coordinate elevation in the E3 ligases atrogin-1 and muscle RING-finger protein-1 (MuRF1). These changes were associated with increased regulated in development and DNA damage response 1 (REDD1) and phosphorylation of AMP-activated protein kinase (AMPK) but no increase in AKT or forkhead transcription factor (FOXO)3 phosphorylation. Finally, markers of autophagy (e.g., LC3B, Atg7, Atg12) and TNF-α were increased to a greater extent in alcohol-fed aged rats. These data demonstrate that aged female rats exhibit an enhanced sensitivity to alcohol compared with adult animals. Our data are consistent with a model whereby alcohol increases proteolysis via FOXO-independent increase in atrogin-1, which degrades eIF3f and therefore impairs formation of a functional preinitiation complex and protein synthesis.

protein synthesis; protein degradation; mTOR; eIF3; atrophy; heart

EXCESSIVE CONSUMPTION OF ALCOHOL (ethanol) remains a major societal issue and detrimentally impacts all homeostatic systems (e.g., hormonal, nervous, and immune) and a diverse array of tissues. Whereas the effect of alcohol on cardiovascular mortality is J-shaped, with relatively low- to moderate-consumption potentially having beneficial effects (8), excessive alcohol intake of long duration has well-recognized deleterious effects on the heart (44). When the excessive alcohol consumption is longstanding, a nonischemic cardiomyopathy develops, which is referred to as alcoholic cardiomyopathy (59). Whereas the etiology of this distinct disease entity is poorly understood, it is most certainly influenced by both genetic and environmental components (13, 33) and is more symptomatic in individuals where alcohol consumption is increased with regard to amount and duration (71). Gender may also be a factor as women develop an asymptomatic form of alcoholic cardiomyopathy with a lower total lifetime consumption of alcohol (12, 70). In contrast, alcohol-induced myocardial dysfunction appears more severe in male compared with female rats (14, 15), and gender-related differences in cardiac morphology and function have been noted in aged versus adult animals (16). Currently, the overwhelming majority of studies on alcohol-induced changes in heart have been performed in males, thereby potentially limiting our understanding of disease etiology. Moreover, essentially all studies have used relatively juvenile rats, with little consideration given to age. This is potentially important because aging renders the heart more susceptible to various types of ischemic stresses (27, 39) in addition to impairing various aspects of basal myocardial function (38).

Chronic alcohol consumption and acute alcohol intoxication decrease myocardial protein synthesis in male rats, and these alcohol-induced changes in protein homeostasis are associated with impaired cardiac function (14, 35, 41, 75). The reduction in cardiac protein synthesis by alcohol is mediated at least in part by a decreased kinase activity of mammalian target of rapamycin (mTOR), which resides in two multiprotein complexes referred to as mTORC1 and mTORC2. It is mTORC1, transducing signals from growth factors, nutrients, and energy status that predominantly regulates protein synthesis (17). In various catabolic conditions, specific alterations in protein-protein interactions among mTOR, raptor (rapamycin-sensitive adaptor protein of mTOR), and other accessory proteins have been implicated for the changes in muscle protein synthesis (30, 31, 36, 47). Conversely, a change in cardiac mass could also be regulated via alterations in the degradative side of the protein balance equation (60, 82). In this regard, the content of select target proteins within a tissue can be mediated via changes in the ubiquitin (Ub)-proteasome pathway (UPP). This is the predominant nonlysosomal degradative pathway requiring both polyubiquitination of the target proteins via specific
E3 ligases and then their subsequent degradation by the proteasome (9, 49). In striated muscle, the muscle-specific E3 ligases atrogin-1/muscle atrophy F-box (MAFbx) and muscle RING-finger protein-1 (MuRF1) appear to regulate protease degradation (2, 4, 21). In cardiac muscle, both ligases also appear to be critical to regulating cardiomyocyte size and heart mass (2, 29, 50, 79). However, the potential role of these E3 ligases in particular and protein breakdown in general in the etiology of alcoholic cardiomyopathy have not been assessed. Therefore, the purpose of the present study was to determine whether changes in myocardial protein balance, both protein synthesis and degradation, and cardiac function that might be produced by long-term alcohol consumption would differ between adult and aged female rats.

**MATERIALS AND METHODS**

*Animal protocol.* Specific pathogen-free adult (4 mo) and aged (18 mo) female Fischer 344 (F344) rats were purchased from the NIA colony at Tacenic (Hudson, NY). Rats were housed under constant environment conditions and received standard rat chow (LabDiet 5001; PMI Nutrition International, St. Louis, MO) and water ad libitum for at least 1 wk. Rats were then randomized to either an alcohol-fed or control group. Each group was maintained for 20 wk on the Lieber-DeCarli liquid diet (Bio-Serv, Frenchtown, NJ). Rats consuming the ethanol-containing diet initially received 12% of total calories from ethanol, and this percentage was increased weekly by 12% until a maximum of 36% of caloric intake from alcohol was achieved. Time-matched pair-fed control animals received a liquid diet where maltose-dextran was isocalorically substituted for alcohol (67). Consumption of the liquid diet was assessed daily and animals were weighed weekly. This duration of alcohol feeding leads to skeletal muscle wasting and alterations in muscle protein balance in both male and female rats (15, 61, 72, 73, 77). 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 laboratory animals.

*Cardiac protein synthesis.* In vivo-determined myocardial protein synthesis was quantified between 08:00 and 10:00 h in randomized control and alcohol-fed rats using the flooding-dose technique, exactly as described (76). As food was removed at ~0500 h, synthesis was determined 3–5 h after food withdrawal. The carotid artery was catheterized for collection of blood, and a bolus injection of [1-2,3,4,5,6-3H]phenylalanine (Phe; 150 mM, 30 μCi/ml; 1 ml/100 g body wt) was injected percutaneously via the jugular vein. Serial arterial blood samples were collected before and after Phe injection for HPLC measurement of Phe concentration and radioactivity. The heart (right and left ventricle only) was excised and a portion freeze-clamped with the remaining muscle being homogenized. All plasma and heart samples were stored at ~80°C until analyzed. Frozen muscle was powderd under liquid nitrogen and the rate of incorporation of [1H]Phe into myofibrillar and sarcoplasmic proteins determined as previously described (41, 42, 72, 73, 78).

*Estimates of protein degradation.* Heart was homogenized in lysis buffer containing (in mM) 25 HEPES, 5 MgCl2, 5 EDTA, 5 DTT, pH 7.5 at 4°C followed by centrifugation (48). The proteasome enzymatic activity was measured by using a proteasome 20S assay kit (Enzo Life Sciences, Farmingdale, NY) as described (36). Proteasome 20S activity was determined by measuring the hydrolysis of a fluorogenic peptidyl substrate Suc-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin, and the subsequently released free AMC was detected using a fluorometer. The change in fluorescence signal was normalized to the amount of protein (BCA Protein Assay Kit, Pierce, Rockford, IL). Addition of the highly specific 20S proteasomal inhibitor MG132 (Boston Biochem, Cambridge, MA) was used to account for any nonproteasomal degradation. Real-time quantitative PCR was used to quantitate the mRNA content of atrogin-1 and MuRF1 (4, 21), as outlined below.

**Real-time quantitative PCR.** Total RNA was extracted from the heart using Tri-reagent (Molecular Research Center, Cincinnati, OH) and the RNeasy mini kit (Qiagen, Valencia, CA) as per manufactures’ instructions. RNA was eluted from the column with RNase-free water, an aliquot was quantitated (NanoDrop 2000, Thermo Fisher Scientific, Waltham, MA), and the quality was analyzed on a 1% agarose gel. Total RNA (1 μg) was reverse transcribed using superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). 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 (Rn00591730_m1), MuRF1 (Rn00590197_m1), interleukin (IL)-6 (Rn01410330_m1), IL-1β (Rn00567600_m1), tumor necrosis factor (TNF)-α (Rn01525859_g1), nitric oxide synthase (NOS)-2 (Rn00561646_m1), and L32 (Rn00820748_g1). The comparative quantitation method 2-ΔΔCT was used in presenting gene expression of target genes in reference to the endogenous control.

*Western blotting and immunoprecipitation.* Heart was homogenized in ice-cold homogenization buffer consisting of (in mM): 20 HEPES (pH 7.4), 2 EDTA, 50 mM sodium fluoride, 100 potassium chloride, 0.2 EDTA, 50 β-glycerophosphate, 1 DTT, 0.1 phenylmethanesulfonyl fluoride, 1 benzamidine, and 0.5 sodium vanadate and clarified by centrifugation, exactly as described (30, 31, 45, 48). Equal amounts of protein per sample were subjected to SDS-PAGE for total and phosphorylated ribosomal protein S6 kinase 1 (S6K1; Thr389), S6 (S240/244), and eukaryotic initiation factor 4E binding protein-1 (4E-BP1; Ser65; Bethyl Laboratories, Montgomery, TX). In addition, total and Thr246-phosphorylated PRAS40 (proline-rich Akt substrate 40), total and Ser792-phosphorylated Raptor, total and Thr172-phosphorylated 45’-AMP-activated kinase (AMPKα), total and phosphorylated Akt (both Thr308 and Ser473), total and phosphorylated (Thr32) forhead transcription factor (FOXO)-3 as well as total GβL (G protein β-subunit-like protein/mLST8), Deptor (also known as DEP domain containing 6; DEPCD), REDD1 (regulated in development and DNA damage responses; Millipore, Billerica, MA), and eIF3f (Abcam, Cambridge, MA) were also determined by Western analysis. To assess autophagy, Western analysis was performed using antibodies against LC3B, Beclin-1, Atg5, Atg7, and Atg12. Additionally, α-tubulin or eIF4E where used, were appropriate, as loading controls. Unless otherwise indicated, antibodies were obtained from Cell Signaling (Beverly, MA). Blots were developed with enhanced chemiluminescence (ECL) Western blotting reagents and then exposed to X-ray film in a cassette equipped with a DuPont Lightning Plus intensifying screen. The film was scanned (Microtte ScanMaker IV) and analyzed using NIH Image 1.6 software.

The eIF4E-eIF4G complexes were quantified as described (40, 42). Briefly, eIF4E was immunoprecipitated from aliquots of supernatants using an anti-eIF4E monoclonal antibody. Antibody-antigen complexes were collected using magnetic beads, subjected to SDS-PAGE, and finally transferred to a PVDF membrane. Blots were incubated with a mouse anti-human eIF4E antibody, rabbit anti-rat 4E-BP1 antibody, or rabbit anti-eIF4G antibody. Also, to investigate protein-protein interactions within mTORC1, fresh muscle was also homogenized in 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS) buffer (pH 7.5) composed of (in mM): 40 HEPES, 120 NaCl, 1 EDTA, 10 pyrophosphate, 10 β-glycerophosphate, 50 NaF, 1.5 sodium vanadate, 0.3% CHAPS, and 1 protease inhibitor cocktail tablet (31, 47). The homogenate was clarified by centrifugation, an aliquot of the supernatant was combined with either anti-Raptor or anti-eIF3f antibody, and immune complexes were isolated with goat anti-rabbit BioMag IgG (PerSeptive Diagnostics, Boston, MA) beads. The beads were collected, washed with CHAPS buffer, precipitated by centrifugation, and subjected to SDS-PAGE as described above. All blots were then developed with ECL and the autoradiographs were quantified as above.
Echocardiography. Heart function was assessed by echocardiography using the Sequoia C256 Echocardiography System (Siemens Medical Solutions, Mountain View, CA) equipped with a 7.5-MHz transducer, as described (62). Rats were lightly anesthetized by the intraperitoneal injection of the combination of ketamine (40 mg/kg) and xylazine (1 mg/kg), and body temperature was maintained by heating pad. The transducer was placed on the thorax, and M-mode recordings were obtained by directing the ultrasound beam at the midpapillary muscle level. End points were obtained after well-defined, continuous interfaces of the septal and posterior walls were visualized a parasternal short-axis view. The operator was blinded to the treatment group of the experimental animal, and measurements from three to four consecutive cardiac cycles were averaged for all animals.

Statistical analysis. Experimental data are summarized as means ± SE where the number of rats per group is provided in the legend to the figure or table. Statistical evaluation of the data was performed using two-way analysis of variance with post hoc Student-Newman-Keuls test. Differences were considered significant when P < 0.05.

RESULTS

Cardiac structure and function. There was no significant difference for any end point assessed by echocardiography between adult rats fed the control and alcohol-containing diet (Table 1, columns 1 and 2). Likewise, echocardiographically-assessed end points for cardiac structure and function did not differ between adult and aged rats fed the control diet (Table 1, columns 1 and 3), although left ventricular diastolic volume (LVDV) and LV diastolic dimension (LVDd) tended (P = 0.08–0.09) to be increased in aged compared with adult, control-fed rats (Table 1). While the ventricle (right + left) mass was increased in control-fed aged rats compared with adult animals, this increase was proportional to the increased body weight (BW); as a result the ventricular mass-to-BW ratio did not differ between adult and aged control-fed rats. In contrast, LV dysfunction was observed in alcohol-fed aged rats (Table 1, columns 3 and 4). For example, selected end points related to myocardial function were decreased in alcohol-fed aged rats including stroke volume (SV; −30%), LVDV (−43%), and the percent ejection fraction (EF%; −30%). Similarly, alcohol-fed aged rats had decreased ventricular mass (−29%), LVDd (−45%), posterior wall thickness during diastole (PWTd; −45%), posterior wall thickness during systole (PWTs; −58%), and percent fractional shortening (FS%; −43%), suggesting altered myocardial structure.

Protein synthesis and mTOR regulation. As with the echocardiography data described above, there were few, if any, significant differences for the various metabolic endpoints assessed for either: 1) control-fed versus alcohol-fed adult rats and 2) adult versus aged control-fed rats. Hence, this section and the DISCUSSION primarily focus on describing the significant changes detected in alcohol-fed aged rats.

In vivo-determined myocardial protein synthesis was reduced 30% in alcohol-fed aged rats compared with all other groups (Fig. 1). This reduction in global protein synthesis resulted from a coordinate alcohol-induced decrease in the synthesis of both myofibrillar and sarcoplasmic proteins (−30% and 40%, respectively).

Figure 2 illustrates that T389-phosphorylated S6K1 and S65-phosphorylated 4E-BP1, known downstream substrates of mTOR (17), were both reduced >50% in hearts from alcohol-fed aged rats. Phosphorylation of the ribosomal protein S6, an endogenous substrate for S6K1, was also decreased in these hearts (Fig. 3). The alcohol-induced decrease in 4E-BP1 phosphorylation would be expected to impair cap-dependent translation by shifting the distribution of eIF4E between the active eIF4AG1-eIF4E to the inactive 4E1BP1-eIF4E cap-binding complex (24). Consistent with the decreased 4E-BP1 phosphorylation, there was a 70% reduction in the binding of eIF4G to eIF4E in hearts from alcohol-fed aged rats (Fig. 3), although the total amount of eIF4G did not differ.

mTOR complex 1 (mTORC1) consists of the major scaffolding protein Raptor as well as GβL, PRAS40, and Deptor (17). In this regard, neither age nor alcohol altered the cardiac content of mTOR, Raptor, GβL, PRAS40, or Deptor (Fig. 4). Raptor functions as a scaffold protein recruiting substrates to mTORC1 (25). When Raptor was immunoprecipitated and mTOR immunoblotted, the extent of mTOR-Raptor binding in heart was increased in adult alcohol-fed rats and tended to be elevated in alcohol-fed aged animals (Fig. 4). However, there was no increase in the binding of Deptor, a

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<th>Table 1. Body weight and echocardiographic assessment of myocardial structure and function in alcohol-fed adult and aged rats</th>
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Values are means ± SE; n = 8–9 rats per group. BW, body weight; LVM, left ventricular mass. Values with different letters in the same row are statistically different from each other (P < 0.05).
known negative regulator of mTOR, to Raptor in the immunoprecipitate (Fig. 4).

The total amount of eIF3f, which is essential for the formation of a functional preinitiation complex, was decreased more than 50% in alcohol-fed age rats compared with all other groups (Fig. 5). Furthermore, when an equal amount of eIF3 was immunoprecipitated from hearts in each group, there was a decreased formation of both the eIF3·Raptor complex and eIF3·eIF4G complex in aged rats consuming alcohol (Fig. 5).

**Protein breakdown.** When compared with adult control-fed rats, hearts from aged control-fed animals demonstrated a small, albeit statistically significant, reduction in both atrogin-1 and MuRF1 mRNA content (Fig. 6). Furthermore, this age-related decrease was associated with a coordinate decrease in in vitro-determined proteasome activity (Fig. 6). In contrast, proteasome activity was increased ~40% in hearts from alcohol-consuming aged rats compared with adult rats (Fig. 6).

Likewise, the myocardial mRNA content for both atrogin-1 and MuRF1 was increased in alcohol-fed aged rats (~6-fold and 2-fold, respectively) (Fig. 6).

Alcohol consumption appeared to increase autophagy signaling in both adult and aged rats (Fig. 7). For example, both the cytosolic form of LC3B-I and the lipidated membrane-bound isoform LC3B-II were increased to the same extent by alcohol consumption. However, as a result of these coordinate changes, the LC3B-II-to-LC3B-I ratio did not differ between groups. The Atg7 protein, which initiates the conjugation of Atg12 to Atg5 and LC3B to phospatidylethanolamine, was increased by alcohol feeding in adult rats and further increased.
in aged rats consuming alcohol. However, Atg12 was only increased in alcohol-fed aged rats compared with all other groups. In contradistinction, Beclin-1 content was not altered by either age and/or alcohol consumption in female rats.

Potential regulators of protein synthesis and degradation. The Thr172-phosphorylation of AMPK was increased 40% in hearts from alcohol-fed adult rats, but a threefold increase was observed in aged rats fed alcohol (Fig. 8). In general, this alcohol-induced change in AMPK phosphorylation was consistent with the increased phosphorylation of LKB1, the upstream activator of AMP (data not shown). Although the phosphorylation of Raptor on Ser792 is AMPK dependent (23), only alcohol-fed aged rats had a significant increase in Raptor phosphorylation (Fig. 8). Finally, REDD1 is a stress-response gene activated by AMPK (11) and was increased more than threefold in hearts from alcohol-fed adult rats and sixfold in alcohol-fed aged rats (Fig. 8).

There was no significant difference in Thr308- or S473-phosphorylation of Akt, a positive regulator of mTORC1 and a negative regulator of proteolysis (18), among the four experimental groups (data not shown). Similarly, the phosphorylation of a downstream substrate for Akt, PRAS40, also did not differ between groups (data not shown). Phosphorylation of FOXO proteins can inhibit atrogin-1 and MuRF1 expression in a number of experimental systems (81). However, again, no significant alcohol- and/or age-induced change in myocardial FOXO3 phosphorylation was detected (data not shown).

Table 2 presents RT-PCR data indicating that neither chronic alcohol feeding nor aging altered IL-1β or IL-6 mRNA.
content in heart. TNF-α mRNA content was greater in aged compared with adult hearts from control-fed rats. Alcohol feeding increased NOS2 and TNF-α mRNA in both adult and aged rats. While the alcohol-induced increase in NOS2 was comparable (3-fold) between adult and aged rats, the TNF-α increased was significantly greater in the later group.

**DISCUSSION**

Multiple lines of evidence indicate alcohol consumption for a period of ~12–24 wk in male rats reduces myocardial protein synthesis (15, 61, 69, 72, 73, 75, 77). This alcohol-induced decrement is in part mTOR-dependent as demonstrated by the reduced phosphorylation of mTOR, S6K1, S6, eIF4G, and 4E-BP1, the latter of which is responsible for the observed reduction in the assembly of the active eIF4E-eIF4G cap-binding complex (72, 73, 77). These protein metabolic effects are also associated with a cardiac dysfunction after longer periods of alcohol consumption (14, 35, 64, 66). However, in our current study, alcohol feeding for 20 wk did not alter the rate of global, myofibrillar, or sarcoplasmic protein synthesis in female F344 rats. Furthermore, cardiac function assessed by echocardiography was also not consistently altered in adult alcohol-fed female rats. These data are consistent with those previously reported in adult female Sprague-Dawley rats by Vary et al. (75), but contrast with those in humans where women appear to be more sensitive to the toxic effects of alcohol (70). It is noteworthy, that such data do not exclude the possibility that alcohol decreased (or even increased) the synthesis of specific relatively low abundance proteins in heart (14, 15). As the focus of the current study was on the interaction of alcohol and aging, the underlying mechanism for this sexual dimorphic response in cardiac protein metabolism to alcohol was not further investigated. However, sexual dimorphic cardiovascular differences are observed under many physiological and pathophysiological conditions (57). While independent lines of evidence suggest estrogen regulates a diverse array of important cardiovascular sex differences, progesterone, androgens, and other hormones (e.g., renin-angiotensin) may also influence heart and vascular function. Moreover, there is a growing literature suggesting that estrogen in particular can mediate sex differences in cardiovascular function via both nuclear estrogen receptor (ER)-α and -β as well as via membrane-associated ERs including G protein-coupled estrogen receptor 1 (GPER) (1). The relative importance of these receptors and individual signal transduction pathways as the cellular basis for the cardiovascular sex differences to alcohol will require additional research using established approaches (54).

Cardiac dysfunction is also a common manifestation of aging in humans and rats (38). However, in the current study, the effect of aging per se on myocardial structure, function, and protein metabolism under basal conditions in female F344 rats...
was nominal. These data differ from those reported by Boluyt et al. (5) in which female F344 rats showed a more marked age-associated increase in LV end-diastolic and end-systolic volume and dimension and decrease in fractional shortening and ejection fraction. There are two major differences between this and the current study. First, in Boluyt et al. (5), changes were detected when aged rats were compared with 4-mo-old animals, whereas our comparison group of adult rats were 4 mo of age when placed on the alcohol-containing diet but 8 mo of age when cardiovascular and metabolic endpoints were assessed. Moreover, the cardiac dysfunction noted by Boluyt et al. (5) was more pronounced at 30 mo compared with 22 mo, whereas our comparison group of adult rats were 4 mo of age when placed on the alcohol-containing diet but 8 mo of age when cardiomyopathy produced by dexamethasone as well as the regression of pressure overload hypertrophy (29, 79). In alcohol-fed adult rats both atrogin-1 and MuRF1 expression in cardiac tissue was nominal. These data differ from those reported by Boluyt et al. (5) in which female F344 rats showed a more marked age-associated increase in LV end-diastolic and end-systolic volume and dimension and decrease in fractional shortening and ejection fraction. There are two major differences between this and the current study. First, in Boluyt et al. (5), changes were detected when aged rats were compared with 4-mo-old animals, whereas our comparison group of adult rats were 4 mo of age when placed on the alcohol-containing diet but 8 mo of age when cardiovascular and metabolic endpoints were assessed. Moreover, the cardiac dysfunction noted by Boluyt et al. (5) was more pronounced at 30 mo compared with 22 mo, the time point used in our current study. Hence, the conclusion reached may depend not only on the absolute age of the old animals, but also on the age of the adult rats to which they are compared. Second, we cannot exclude the possibility that differences in dietary composition between studies may impact age-associated changes. Whereas both studies provided a diet with the same protein content (~28%), rats in the Boluyt study received ~13% of their total calories from fat (via traditional rodent chow) while our rats were fed a nutritionally complete liquid diet containing ~30% fat. While this relatively high-fat diet is a standard model in alcohol research (51), it may nonetheless modify the response to aging. Hence, our data are more consistent with previous findings in isolated perfused hearts that showed no change in baseline systolic function in the aged rat (28).

Likewise, our assessment of in vivo-determined cardiac protein synthesis revealed no difference between adult versus aged rats fed the control diet. These results are internally consistent with the lack of change in the phosphorylation state of various proteins both up- and downstream of mTOR. These data are also consistent with reports indicating cardiac protein synthesis did not differ in either male F344 or Sprague-Dawley rats that were the same age as those used in the current study (3, 20) but do differ from the decreased myocardial protein synthesis observed in other studies (10). In contrast, an age-related decrease in vitro-determined proteasome activity was detected in control-fed rats. Although such a decrement has been previously reported (6, 32), this is the first report of a coordinated age-related decrease in both atrogin-1 and MuRF1 expression in cardiac tissue.

Based on the reduced tolerance of hearts from aged animals to stress conditions, such as inflammation, ischemia-reperfusion, and hypoxia, (39, 52, 55, 65), we hypothesized that chronic alcohol ingestion would exacerbate age-related changes in myocardial structure, function, and protein balance. Indeed, hearts from alcohol-fed aged rats exhibited a relative atrophy, as evidenced by the reduced left ventricular mass and posterior wall thickness compared with control-fed rats of the same age. Myocardial dysfunction in these rats was evidenced by the reduced SV, CI, LV diastolic volume, EF%, and FS%. These structural and functional changes in alcohol-fed rats were associated with decreases in protein synthesis and increases in protein degradation, changes not detected in response to either aging or alcohol consumption alone. Although we interpret these data to suggest hearts from aged rats have an increased sensitivity or reduced tolerance to the deleterious cardiac effects of chronic alcohol consumption, similar to that seen in response to other stressors, such a conclusion is not equivocal because of the nominal changes produced by either aging or alcohol alone. Alternatively, it is possible the current data indicate the interaction of alcohol and aging in female rats induces specific effects not produced by either variable alone.

Atrogin-1 and MuRF1 are ubiquitin E3 ligases that are coordinately upregulated in many catabolic conditions, and the involvement of these “atrogenes” and the UPP in regulating protein degradation in striated muscle is clearly established (4, 21, 37, 46, 48). While alcohol has been reported to increase these ligases in skeletal muscle (58, 74), no data are available regarding cardiac atrogene expression in response to alcohol feeding. Ubiquitin ligases confer specificity to the system by the selective ubiquitination of target proteins, which are then degraded by the proteasome. For example, cardiocpecific overexpression of atrogin-1 antagonizes the development of cardiac hypertrophy toward various stimuli (50). Conversely, MuRF1 is causally related to the cardiac atrophy produced by dexamethasone as well as the regression of pressure overload hypertrophy (29, 79). In alcohol-fed adult rats both atrogin expression and proteasome activity were
unchanged compared with age-matched control-fed rats. However, 20S proteasome activity was decreased in hearts from aged rats, a response previously reported (32), and this associated with a decreased content of atrogin-1 and MuRF1 mRNA. In contradistinction, there was a coordinate increase in both proteasome activity and atrogene mRNA content in hearts from alcohol-fed aged rats. Although both atrogin-1 and MuRF1 were increased in these rats, the increment in atrogin-1 predominated. This finding is potentially revealing because the initiation factor eIF3f, which was decreased in hearts from alcohol-fed aged rats, is a known substrate for atrogin-1 and a reduction in eIF3f has been reported in skeletal muscle atrophy (9). Furthermore, the increased mRNA expression of these atrogens was associated with an overall increase in proteasome activity, which would be expected to enhance global myocardial protein degradation.

Fig. 7. Effect of chronic alcohol feeding on autophagy-related signaling protein expression in hearts from adult and aged female F344 rats. Top left, representative Western blots for LC3B-I and -II, Beclin-1, Atg7, and Atg12; α-tubulin used as loading control. Bar graphs, quantitation of immunoblot data normalized for loading protein with values from control-fed adult rats set at 100 AUs. Values are means ± SE, n = 8–9 rats per group. Groups with different superscript letters are significantly different from each other (P < 0.05).

Fig. 8. Effect of chronic alcohol feeding on relative content of total and phosphorylated AMP-activated protein kinase (AMPK) and Raptor, and total regulated in development and DNA damage response 1 (REDD1) in hearts from adult and aged female F344 rats. Top left, representative Western blot for the phosphorylated protein of interest and total protein used as loading control. Bar graphs, quantitation of immunoblot data normalized for loading protein with values from control-fed adult rats set at 100 AUs. Values are means ± SE, n = 8–9 rats per group. Groups with different superscript letters are significantly different from each other (P < 0.05).
Additionally, autophagy is a survival pathway that contributes to intracellular degradation of protein and organelles permitting cells to maintain energy homeostasis. In the heart, autophagy is activated during various stress conditions, including exposure to TNF-α (82). Although this pathway was not investigated in depth, several key regulators of autophagy, such as LC3B-I and -II, Agt7, and Agt12, were increased in hearts from alcohol-fed aged rats. Increased cardiac autophagy has also been reported in alcohol-fed mice (22). The induction of the UPP and autophagy is tightly controlled by a host of regulators and of these the Akt-FoxO signaling pathway is arguably the most important (7, 68, 81). Inhibition of Akt and dephosphorylation of FOXO leads to nuclear entry and transcription of ubiquitin ligases as well as activation of autophagy. However, this mechanism does not appear operational in the current study as the phosphorylation of both Akt and FoxO3 was unchanged in hearts from alcohol-fed aged rats. AMPK stimulation represents another means to increase atrogin-1 in heart (2), and our current data showing the concomitant phosphorylation of LKB, AMPK, and Raptor support the activation of this pathway in hearts of alcohol-fed aged rats. While cardiac autophagy appears increased in alcohol-fed aged rats, it is unclear whether this increase is dependent (either directly or indirectly) on the coordinate decrease in protein synthesis or, alternatively, is a reflection of the extensive and overlapping nature inherent in mTOR signal transduction pathways.

Conversely, alcohol consumption by aged rats also decreased myocardial protein synthesis. The reduced phosphorylation of 4E-BP1 and S6K1 is indicative of impaired mTOR kinase activity, which again appears to be Akt independent. The reduced phosphorylation of 4E-BP1 was associated with a decreased formation of the active cap-binding complex eIF4E-eIF4G. Moreover, the total amount of eIF3f, one component of a multisubunit protein complex, which serves as a docking site for the binding of several proteins on the translational machinery (26), was dramatically reduced in hearts from alcohol-fed aged rats. These rats had reduced binding of both Raptor and eIF4G to eIF3, suggesting impaired binding of this complex with mRNA and to the 43S ribosomal complex. While these data suggest a putative mechanism for the decrease in cardiac protein synthesis, no such change in eIF3 content or protein-protein binding were reported in skeletal muscle from alcohol-fed aged rats (36). Hence, the deleterious effects of chronic alcohol consumption on heart and skeletal muscle appear mediated by different cellular mechanisms. Because protein synthesis is also inhibited by AMPK activation (63) and increased REDD1 (11), we cannot exclude these possible mechanisms. Finally, altered binding of raptor to mTOR can impair mTOR kinase activity (17). The increased formation of the mTOR-raptor complex in response to alcohol consumption was associated with the decrease in myocardial protein synthesis. Such changes may suggest alcohol impairs mTOR kinase activity by promoting a closed conformation which renders the kinase less active (34). Regardless of the exact mechanism, the alcohol-induced increase in mTOR-raptor binding was comparable between adult and aged rats and therefore an unlikely mechanism for the exaggerated reduction in cardiac protein synthesis in alcohol-fed aged rats.

The chronic consumption of alcohol increased cardiac TNF-α mRNA regardless of the age of the rat; however, the elevation in TNF-α was significantly enhanced in the alcohol-fed aged rats. The effect of alcohol and aging on proinflammatory cytokine expression in the heart was not a generalized response as IL-1β and IL-6 mRNAs were not elevated. We also examined NOS2 expression because upregulation of NOS2 and the overproduction of nitric oxide have been implicated as causative in the development and/or progression of cardiomyopathy of various etiologies (56), and because TNF-α-induced increases in NOS2 also inhibit protein synthesis in striated muscle (19). However, while our data show an alcohol-induced increase in NOS2 mRNA in hearts from both adult and aged rats, NOS2 was not further increased in alcohol-fed aged rats suggesting a limited role of this mediator in the observed functional and metabolic changes. Hence, although this study does not allow us to conclude whether the exaggerated increase in TNF-α in alcohol-fed aged rats was causally related to the impaired myocardial protein balance and function observed in these rats, overproduction of TNF-α has been reported to decrease protein synthesis (43) as well as increase proteolysis (53) and autophagy (80) in heart.

**Perspectives and Significance**

The data presented in the current study demonstrate an age-dependent vulnerability of the heart with respect to structure, function, and protein balance in response to chronic alcohol consumption. Alcohol-fed aged rats had a cardiac atrophy that was associated with an upregulation of the UPP and autophagy but also a downregulation of protein synthesis. We posit that AMPK activation increases the UPP in general and the E3 ligase atrogin-1 in particular thereby enhancing eIF3f degradation. The reduced protein abundance of this translation initiation factor, alone or in combination, with the reduced formation of the eIF4E-eIF4G complex then impairs cap-dependent protein translation and protein synthesis of both sarcoplasmic and myofibrillar proteins. These data provide a contextual framework for the reduced cardiac tolerance to chronic alcohol consumption in aged female F344 rats. It is unknown whether such cardiac defects are also manifested in aged male rats following chronic alcohol ingestion or whether the observed alterations in cardiac function and protein metabolism are gender specific. Despite differences between the currently used rodent model of chronic alcohol feeding and the sustained abuse of alcohol by humans, the blood alcohol concentration (100–200 mg/dl) achieved in rats and humans is comparable supporting the translational relevance of our ob-

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<td>TNF-α</td>
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<td>IL-1β</td>
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Values are means ± SE expressed as AU/L32; <sup>a</sup>n = 8–9 rats per group. L32 was used for normalization and was not statistically different between groups. Values with different letters in the same row are statistically different from each other (P < 0.05).
servations. While ischemic heart disease is a more prevalent cause of heart damage relatively to alcohol abuse, the later induces a specific type of cardiomyopathy and sheds light on potential mechanisms. Collectively, our data suggest that sustained excessive alcohol consumption by elderly females should be discouraged to reduce the likelihood and/or minimize the development of potentially deleterious changes in cardiac structure, function, and metabolism.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: C.H.L. conception and design of research; C.H.L. performed experiments; C.H.L. analyzed data; C.H.L. and D.H.K. interpreted results of experiments; C.H.L. prepared figures; C.H.L. and D.H.K. drafted manuscript; C.H.L. and D.H.K. edited and revised manuscript; C.H.L. and D.H.K. approved final version of manuscript.

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