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

Charles H. Lang¹,²
Donna H. Korzick²,³

Department of Cellular & Molecular Physiology,¹ and InterCollege Graduate Program in Physiology²
Penn State College Medicine, Hershey, PA 17033, and
Department of Kinesiology³,
The Pennsylvania State University, University Park, PA 16802

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Correspondence to:
Charles H. Lang, PhD
Cell & Molec Physiology
Penn State College Medicine
500 University Drive
Hershey, PA 17033
phone: 717-531-5538
fax: 717-531-7667
clang@psu.edu
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 m) and aged (18 m) F344 rats were fed a nutritionally complete liquid diet containing alcohol (36% total calories) or an isocaloric isonitrogenous control diet for 20 wks. Cardiac structure and function, assessed by echocardiography, as well as myocardial protein synthesis and proteolysis did not differ in either alcohol- vs control-fed adult rats or in adult vs aged control-fed rats. In contrast, cardiac function was impaired in alcohol-fed aged rats, compared to age-matched control rats. Additionally, alcohol feeding decreased cardiac protein synthesis which was associated with decreased phosphorylation of 4E-BP1 and S6K1. This reduction in 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 MuRF1. These changes were associated with increased REDD1 and phosphorylation of AMPK, but no increase in AKT or FOXO3 phosphorylation. Finally, markers of autophagy (e.g., LC3B, Atg7, Atg12) and TNFα 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 to 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 pre-initiation complex and protein synthesis.

Key words: 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. While 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 non-ischemic cardiomyopathy develops which is referred to as alcoholic cardiomyopathy (59). While 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 to 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 mTOR
(mammalian target of rapamycin), which resides in two multi-protein complexes referred to as mTORC1 and mTORC2. It is mTORC1, transducing signals from growth factors, nutrients and energy status, which predominantly regulates protein synthesis (17). In various catabolic conditions, specific alterations in protein-protein interactions between 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 poly-ubiquitination 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 MuRF1 (muscle RING-finger protein-1) appear to regulate protein degradation (2, 4, 21). In cardiac muscle, both ligases also appear to be critical to regulating cardiomyocyte size and heart muscle 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 which 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 month) and aged (18 month) female Fischer 344 (F344) rats were purchased from the NIA colony at Taconic (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 wks 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% till 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 experimental animals.

**Cardiac protein synthesis.** In vivo-determined myocardial protein synthesis was quantified between 0800 and 1000 hours in randomized control and alcohol-fed rats using the flooding-dose technique, exactly as described (76). As food was removed at approximately 0500 hours, synthesis was determined 3-5 hours after food withdrawal. The carotid artery was catheterized for collection of blood and a bolus injection of L-[2,3,4,5,6-3H]phenylalanine (Phe; 150 mM, 30 μCi/ml; 1 ml/100 g body weight [BW]) 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 powdered under liquid nitrogen and the rate of incorporation of [3H]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, New York) as described (36). Proteasome 20S activity was determined by measuring the hydrolysis of a fluorogenic peptidyl substrate Suc-Leu-Leu-Val-Tyr-AMC (AMC: 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 proteasome 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 heart using Tri-reagent (Molecular Research Center, Inc., 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 analyzed on a 1% agarose gel. Total RNA (1 µg) was reversed 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 (Rn00566700_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 mmol/L) 20 HEPES (pH 7.4), 2 EGTA, 50 sodium
fluoride, 100 potassium chloride, 0.2 EDTA, 50 \( \beta \)-glycerophosphate, 1 DTT, 0.1 phenylmethane-sulphonylfluoride, 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 5'-AMP-activated kinase (AMPK\( \alpha \)), total and phosphorylated Akt (both Thr308 and Ser473), total and phosphorylated (Thr32) forkhead transcription factor (FOXO)-3 as well as total G\( \beta \)L (G-protein \( \beta \)-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 elf3f (Abcam, Cambridge, MA) were also determined by Western analysis. To assess autophagy, Western analysis was performed using antibodies against LC3B, beclin-1, Atg7 and Atg12. Additionally, \( \alpha \)-tubulin or elf4E 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 (Microtek ScanMaker IV) and analyzed using NIH Image 1.6 software.

The elf4E•elf4G complexes were quantified as described (40, 42). Briefly, elf4E was immunoprecipitated from aliquots of supernatants using an anti-elf4E 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 elf4E antibody, rabbit anti-rat 4E-BP1 antibody, or rabbit anti-elf4G antibody. Also, to investigate protein-protein interactions within mTORC1, fresh muscle was also homogenized in
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 and 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 maintained by heating pad. The transducer was placed on the thorax and M-mode recordings obtained by directing the ultrasound beam at the midpapillary muscle level. Endpoints 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 ± standard error of the mean (SEM) 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 2 way-analysis of variance with post-hoc Student-Neuman-Keuls test. Differences were considered significant when $P < 0.05$. 


RESULTS

Cardiac structure and function. There was no significant difference for any endpoint assessed by echocardiography between adult rats fed the control and alcohol-containing diet (Table 1, columns 1 and 2). Likewise, echocardiographic-assessed endpoints 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 to adult, control-fed rats (Table 1). While the ventricle (right + left) mass was increased in control-fed aged rats, compared to adult animals, this increase was proportional to the increased body weight (BW); as a result the ventricular mass/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 endpoints 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: a) control-fed versus alcohol-fed adult rats and b) 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 to all other groups (Figure 1). This reduction in global protein synthesis resulted
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 (Figure 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 eIF4G•eIF4E to the inactive 4EBP1•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 (Figure 3), although the total amount of eIF4 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 (Figure 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 (Figure 4). However, there was no increase in the binding of Deptor, a known negative regulator of mTOR, to Raptor in the immunoprecipitated (Figure 4).

The total amount of eIF3f, which is essential for the formation of a functional pre-initiation complex, was decreased more than 50% in alcohol-fed age rats, compared to all other groups (Figure 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 (Figure 5).
**Protein breakdown.** Compared to 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 (Figure 6). Furthermore, this age-related decrease was associated with a coordinate decrease in in vitro-determined proteasome activity (Figure 6). In contrast, proteasome activity was increased ~40% in hearts from alcohol consuming aged rats, compared to adult rats (Figure 6). Likewise, the myocardial mRNA content for both atrogin-1 and MuRF1 was increased in alcohol-fed aged rats (approximately 6-fold and 2-fold, respectively) (Figure 6).

Alcohol consumption appeared to increase autophagy signaling in both adult and aged rats (Figure 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/-I ratio did not differ between groups. The Atg7 protein, which initiates the conjugation of Atg12 to Atg5 and LC3B to phosphatidylethanolamine, 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 to 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 3-fold increase was observed in aged rats fed alcohol (Figure 8). In general, this alcohol-induced change in AMPK phosphorylation was consistent with the increased phosphorylation of LKB1, the up-stream 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 (Figure 8). Finally, REDD1 is a stress-response gene activated by AMPK (11)
and was increased more than 3-fold in hearts from alcohol-fed adult rats and 6-fold in alcohol-fed aged rats (Figure 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. TNFα mRNA content was greater in aged compared to adult hearts from control-fed rats. Alcohol feeding increased NOS2 and TNFα mRNA in both adult and aged rats. While the alcohol-induced increase in NOS2 was comparable (3-fold) between adult and aged rats, the TNFα increased was significantly greater in the later group.

**DISCUSSION**

Multiple lines of evidence indicate alcohol consumption for a period of approximately 12-24 wks 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 wks 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 
a 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 to 4-month old animals, whereas our comparison group of adult rats were 4 months of age when placed on the alcohol-containing diet but 8 months of age when cardiovascular and metabolic endpoints were assessed. Moreover, the cardiac dysfunction noted by Boluyt et al (5) was more pronounced at 30 months compared to 22 months, 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 which 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 down-stream of mTOR. These data are also consistent with reports indicating cardiac protein synthesis did not differ in either male F344 or Sprague-Dawley rats which 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 decreased 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 LV mass and posterior wall thickness, compared to 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 which are coordinately up-regulated 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, cardiac-specific overexpression of atrogin-1 antagonizes the development of cardiac hypertrophy towards 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 atrogene expression and proteasome activity were unchanged compared to 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 atrogenes was associated with an overall increase in proteasome activity which would be expected to enhance global myocardial protein degradation.

Additionally, autophagy is a survival pathway which 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 TNFα (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
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 multi-subunit 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. As 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 TNFα mRNA regardless of the age of the rat; however, the elevation in TNFα was significantly enhanced in the alcohol-fed
aged rats. The effect of alcohol and aging on proinflammatory cytokine expression in 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 over-production of nitric oxide have been implicated as causative in the development and/or progression of cardiomyopathy of various etiologies (56), and because TNFα-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 TNFα in alcohol-fed aged rats was causally related to the impaired myocardial protein balance and function observed in these rats, overproduction of TNFα 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 which was associated with an up-regulation of the UPP and autophagy but also a down-regulation 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 observations. 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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GRANTS

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FIGURE LEGENDS

Figure 1. Effect of chronic alcohol feeding on in vivo-determined rates of protein synthesis in hearts from adult and aged female F344 rats. Top panel, global protein synthesis; middle panel, sarcoplasmic protein synthesis; and bottom panel, myofibrillar protein synthesis. Values are means ± SEM, n = 8-9 rats per group. Groups with different superscript letters are significantly different from each other (P < 0.05).

Figure 2. Effect of chronic alcohol feeding on the relative content of total and phosphorylated ribosomal S6 kinase (S6K)-1 and eukaryotic initiation factor (eIF) 4E binding protein (4E-BP)-1 in hearts from adult and aged female F344 rats. Top panel, representative Western blots for the phosphorylated protein of interest and total protein used as loading control. Bar graphs, quantitation of immunoblot data normalized for loading protein and values from control-fed adult rats set at 100 arbitrary units (AUs). Values are means ± SEM, n = 8-9 rats per group. Groups with different superscript letters are significantly different from each other (P < 0.05).

Figure 3. Effect of chronic alcohol feeding on the relative content of total and phosphorylated ribosomal S6 kinase (S6) and binding of eIF4G to eIF4E in hearts from adult and aged female F344 rats. Top panel, representative Western blots for phosphorylated and total S6. Also shown a representative blot where eIF4E was immunoprecipitated (IP) from heart homogenates and immunoblotted for eIF4G or eIF4E. Bar graphs, quantitation of immunoblot data normalized for loading protein with values from control-fed adult rats set at 100 AUs. Values are means ± SEM, n = 8-9 rats per group. Groups with different superscript letters are significantly different from each other (P < 0.05).
**Figure 4.** Effect of chronic alcohol feeding on the relative content of various mTOR complex (mTORC)-1 proteins and the binding of mTOR with Raptor. *Top panel,* representative Western blots for total mTORC1 proteins. There were no age- and/or alcohol-induced changes in the relative content for any of these proteins (P = NS; quantitation not shown). *Middle panel,* representative blot where Raptor was immunoprecipitated (IP) from heart homogenates and immunoblotted for mTOR, Raptor or Deptor. Bar graphs, quantitation of immunoblot data normalized for loading protein with values from control-fed adult rats set at 100 AUs. Values are means ± SEM, n = 8-9 rats per group. Groups with different superscript letters are significantly different from each other (P < 0.05).

**Figure 5.** Effect of chronic alcohol feeding on total eIF3f and binding of Raptor and eIF4G to eIF3 in hearts from adult and aged female F344 rats. *Right panel (top),* representative Western blot for total eIF3f and eIF4G where eIF4E was used as a loading control; *right panel (bottom),* eIF3 was immunoprecipitated (IP) from heart homogenates and then Raptor, eIF4G or eIF3f was immunoblotted. Bar graphs, quantitation of immunoblot data normalized for loading protein with values from control-fed adult rats set at 100 AUs. Values are means ± SEM, n = 8-9 rats per group. Groups with different superscript letters are significantly different from each other (P < 0.05).
Figure 6. Effect of chronic alcohol feeding on mRNA content for atrogin-1 and MuRF1 as well as proteasome activity in hearts from adult and aged female F344 rats. Top and middle panels, mRNA data were determined by qRT-PCR, data normalized to L32 and the value from the control-fed adult rats set at 1.0 AU. Bottom panel, in vitro-determined proteasome activity. Values are means ± SEM, n = 8-9 rats per group. Groups with different superscript letters are significantly different from each other (P < 0.05).

Figure 7. Effect of chronic alcohol feeding on autophagy-related signaling protein expression in hearts from adult and aged female F344 rats. Top left panel, 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 ± SEM, n = 8-9 rats per group. Groups with different superscript letters are significantly different from each other (P < 0.05).

Figure 8. Effect of chronic alcohol feeding on relative content of total and phosphorylated AMPK and Raptor, and total REDD1 in hearts from adult and aged female F344 rats. Top left panel, 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 ± SEM, n = 8-9 rats per group. Groups with different superscript letters are significantly different from each other (P < 0.05).
Table 1: Body weight and echocardiographic assessment of myocardial structure and function in alcohol-fed adult and aged rats

<table>
<thead>
<tr>
<th></th>
<th>Adult</th>
<th>Alcohol</th>
<th>Aged</th>
<th>Alcohol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (BW), g</td>
<td>285 ± 7\text{a}</td>
<td>265 ± 6\text{a}</td>
<td>372 ± 11\text{b}</td>
<td>325 ± 15\text{c}</td>
</tr>
<tr>
<td>Right + Left Ventricular mass (VM), mg</td>
<td>852 ± 81\text{a}</td>
<td>788 ± 73\text{a}</td>
<td>1176 ± 96\text{b}</td>
<td>831 ± 93\text{a}</td>
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<tr>
<td>VM/BW, mg/g</td>
<td>2.99 ± 0.24</td>
<td>2.97 ± 0.32</td>
<td>3.01 ± 0.48</td>
<td>2.56 ± 0.39</td>
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<tr>
<td>Stroke volume (SV), ml</td>
<td>0.50 ± 0.05\text{a}</td>
<td>0.46 ± 0.04\text{a}</td>
<td>0.62 ± 0.06\text{a}</td>
<td>0.31 ± 0.04\text{b}</td>
</tr>
<tr>
<td>LV diastolic volume (LVDV), ml</td>
<td>0.67 ± 0.05\text{a}</td>
<td>0.66 ± 0.09\text{a}</td>
<td>0.81 ± 0.07\text{a}</td>
<td>0.41 ± 0.06\text{b}</td>
</tr>
<tr>
<td>LV systolic volume (LVSV), ml</td>
<td>0.13 ± 0.02</td>
<td>0.13 ± 0.01</td>
<td>0.15 ± 0.02</td>
<td>0.10 ± 0.02</td>
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<tr>
<td>Ejection Fraction (EF)%</td>
<td>85 ± 7\text{a}</td>
<td>83 ± 6\text{a}</td>
<td>83 ± 7\text{a}</td>
<td>59 ± 6\text{b}</td>
</tr>
<tr>
<td>LV diastolic dimension (LVDd), cm</td>
<td>0.67 ± 0.05\text{a}</td>
<td>0.66 ± 0.08\text{a}</td>
<td>0.77 ± 0.09\text{a}</td>
<td>0.42 ± 0.08\text{b}</td>
</tr>
<tr>
<td>LV systolic dimension, (LVDs) cm</td>
<td>0.22 ± 0.03</td>
<td>0.21 ± 0.02</td>
<td>0.25 ± 0.03</td>
<td>0.26 ± 0.04</td>
</tr>
<tr>
<td>Fractional shorting (FS)%</td>
<td>67 ± 8\text{a}</td>
<td>68 ± 7\text{a}</td>
<td>67 ± 9\text{a}</td>
<td>38 ± 5\text{b}</td>
</tr>
<tr>
<td>Posterior wall thickness diastole (LVPWd), cm</td>
<td>0.17 ± 0.01\text{a}</td>
<td>0.15 ± 0.02\text{ab}</td>
<td>0.20 ± 0.02\text{a}</td>
<td>0.11 ± 0.01\text{b}</td>
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<tr>
<td>Posterior wall thickness systole (LVPWs), cm</td>
<td>0.42 ± 0.03\text{a}</td>
<td>0.38 ± 0.05\text{a}</td>
<td>0.40 ± 0.06\text{a}</td>
<td>0.17 ± 0.03\text{b}</td>
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<tr>
<td>Interventricular septum thickness systole (LVSs), cm</td>
<td>0.26 ± 0.04</td>
<td>0.26 ± 0.03</td>
<td>0.29 ± 0.04</td>
<td>0.20 ± 0.02</td>
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</table>

Values are means ± SEM; n = 8-9 rats per group. Values with different letters in the same row are statistically different from each other (P < 0.05).
**Table 2:** Cardiac mRNA content for inflammatory mediators in alcohol-fed adult and aged rats

<table>
<thead>
<tr>
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<th>Adult</th>
<th></th>
<th>Aged</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Alcohol</td>
<td>Control</td>
<td>Alcohol</td>
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<tr>
<td>TNFα</td>
<td>1.00 ± 0.06a</td>
<td>2.67 ± 0.33b</td>
<td>2.34 ± 0.3b</td>
<td>7.48 ± 0.79c</td>
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<tr>
<td>IL-1β</td>
<td>1.00 ± 0.11</td>
<td>0.99 ± 0.15</td>
<td>0.98 ± 0.09</td>
<td>1.14 ± 0.17</td>
</tr>
<tr>
<td>IL-6</td>
<td>1.00 ± 0.22</td>
<td>1.07 ± 0.06</td>
<td>0.87 ± 0.14</td>
<td>1.04 ± 0.12</td>
</tr>
<tr>
<td>NOS2</td>
<td>1.00 ± 0.08a</td>
<td>3.45 ± 0.78b</td>
<td>0.93 ± 0.13a</td>
<td>3.26 ± 0.41b</td>
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</tbody>
</table>

Values are means ± SEM expressed as AU/L32; 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).
REFERENCES


14. **Fogle RL, Hollenbeak CS, Stanley BA, Vary TC, Kimball SR, and Lynch CJ.** Functional proteomic analysis reveals sex-dependent differences in structural and energy-


47. **Lang CH, Pruznak AM, Nystrom GJ, and Vary TC.** Alcohol-induced decrease in muscle protein synthesis associated with increased binding of mTOR and raptor: Comparable effects in young and mature rats. *Nutr Metab (Lond)* 6: 4, 2009.


**Figure 2**

**S6K1**

**Adapted from**

**Con Alc Con Alc**

/ and/or Tubulin

**4EBP1**

**Adapted from**

**Con Alc Con Alc**

/ and/or Tubulin

**control**

**Alcohol-fed**

**Adult**

**Aged**

**S6K1**

**P (T389)**

**S6K1**

**total**

**α-tubulin**

**4EBP1**

**P (S65)**

**4EBP1**

**total**

**S6K1 phosphorylation (T389 AU/total)**

**4EBP1 phosphorylation (S65 AU/total)**
Figure 3

**Western Blot Analysis**

**S6 – P (S240/244)**
- **Adult**
  - Con: Control
  - Alc: Alcohol-fed
- **Aged**
  - Con: Control
  - Alc: Alcohol-fed

**S6 (total)**
- **Adult**
  - Con: Control
  - Alc: Alcohol-fed
- **Aged**
  - Con: Control
  - Alc: Alcohol-fed

**Bar Graph**
- **S6 protein (S240/244 AU/total)**
  - **Adult**
    - Control: 100
    - Alcohol-fed: 125
  - **Aged**
    - Control: 75
    - Alcohol-fed: 50

**IP: eIF4G**
- **Adult**
  - Con: Control
  - Alc: Alcohol-fed
- **Aged**
  - Con: Control
  - Alc: Alcohol-fed

**eIF4E-4G binding (AU/eIF4E)**
- **Adult**
  - Control: 100
  - Alcohol-fed: 125
- **Aged**
  - Control: 75
  - Alcohol-fed: 50

Legend:
- Control
- Alcohol-fed

Note: Letter 'a' indicates a significant difference from control, letter 'b' indicates a significant difference from alcohol-fed.
Figure 4

<table>
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<tr>
<th></th>
<th>Adult</th>
<th>Aged</th>
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<tbody>
<tr>
<td></td>
<td>Con</td>
<td>Alc</td>
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<tr>
<td>mTOR</td>
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<td>Raptor</td>
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<td>GβL</td>
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- **IP: raptor**
  - mTOR
  - Deptor
  - Raptor

- **mTOR-Raptor binding (AU/raptor)**
  - Adult: Control (a), Alcohol-fed (ab)
  - Aged: Control (a), Alcohol-fed (ab)
Figure 5

**Western blot**

### eIF3 Content (AU/eIF4E)
- **Adult**
  - Control: 100
  - Alcohol-fed: 125
- **Aged**
  - Control: 75
  - Alcohol-fed: 100

### eIF3-raptor Binding (AU/eIF3)
- **Adult**
  - Control: 125
  - Alcohol-fed: 100
- **Aged**
  - Control: 75
  - Alcohol-fed: 100

### eIF3-eIF4G Binding (AU/eIF3)
- **Adult**
  - Control: 125
  - Alcohol-fed: 75
- **Aged**
  - Control: 125
  - Alcohol-fed: 75
Figure 7

LC3B-I/LC3-I ratio

<table>
<thead>
<tr>
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<th>Con</th>
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<tr>
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<tr>
<td>LC3B-II</td>
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</tr>
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<td>Atg12</td>
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<tr>
<td>α-tubulin</td>
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</tbody>
</table>

Control          | Alcohol-fed

Adult          | Aged

Atg7 (AU/tubulin)

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</thead>
<tbody>
<tr>
<td>Atg7</td>
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Atg12 (AU/tubulin)

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<tbody>
<tr>
<td>Atg12</td>
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</table>
Figure 8

**Western Blotting Results:**

- **AMPK – P (T172)**
- **AMPK (total)**
- **Raptor – P (S792)**
- **Raptor (total)**
- **REDD1**

**Bar Graphs:**

- **AMPK phosphorylation (T172 AU/total AMPK)**
  - Adult: Control (a), Alcohol-fed (b, c)
  - Aged: Control (a), Alcohol-fed (b, c)

- **Raptor phosphorylation (S792 AU/total raptor)**
  - Adult: Control (a, a)
  - Aged: Control (a, a)

- **REDD1 (AU)**
  - Adult: Control (a), Alcohol-fed (a)
  - Aged: Control (a), Alcohol-fed (a)