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Sex-dependent differences in the regulation of myocardial protein synthesis following long-term ethanol consumption

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Submitted 21 March 2006; accepted in final form 25 August 2006

Vary TC, Kimball SR, Sumner A. Sex-dependent differences in the regulation of myocardial protein synthesis following chronic ethanol consumption. Am J Physiol Regul Integr Comp Physiol 292: R778—R787, 2007. First published August 31, 2006; doi:10.1152/ajpregu.00203.2006.—Chronic heavy alcohol consumption alters cardiac structure and function. Controversies remain as to whether hearts from females respond to the chronic ethanol intake in a manner analogous to males. In particular, sex differences in the myocardial response to chronic alcohol consumption remain unresolved at the molecular level. The purpose of the present set of experiments was to determine whether alterations in cardiac structure and protein metabolism show sexual dimorphism following chronic alcohol consumption for 26 wk. In control animals, hearts from female rats showed lowered heart weights and had thinner ventricular walls compared with males. The smaller heart size was associated with a lower protein content that occurred in part from a reduced rate of protein synthesis. Chronic alcohol consumption in males, but not in females, caused a thinning of the ventricular wall and intraventricular septum, as assessed by echocardiography, correlating with the loss of heart mass. The alterations in cardiac size occurred, in part, through a lowering of the protein content secondary to a diminished rate of protein synthesis. The decreased rate of protein synthesis appeared related to a reduced assembly of active eukaryotic initiation factor (eIF)4E complex secondary to both a diminished phosphorylation of eIF4E and increased formation of inactive 4Ebinding protein (4EBP1)-eIF4E complex. The latter effects occurred as a result of decreased phosphorylation of 4EBP1. None of these ethanol-induced alterations in hearts from males were observed in hearts from females. The smaller heart size was associated with a lower protein content that occurred in part from a reduced rate of protein synthesis. Chronic alcohol consumption in males, but not in females, caused a thinning of the ventricular wall and intraventricular septum, as assessed by echocardiography, correlating with the loss of heart mass. The alterations in cardiac size occurred, in part, through a lowering of the protein content secondary to a diminished rate of protein synthesis. The decreased rate of protein synthesis appeared related to a reduced assembly of active eukaryotic initiation factor (eIF)4E complex secondary to both a diminished phosphorylation of eIF4E and increased formation of inactive 4Ebinding protein (4EBP1)-eIF4E complex. The latter effects occurred as a result of decreased phosphorylation of 4EBP1. None of these ethanol-induced alterations in hearts from males were observed in hearts from females.

Alcoholic heart muscle disease is rarely produced by short-term ethanol administration (71). Instead, the disease occurs in patients in whom the sole causative agent is excessive and prolonged alcohol consumption (>80 g of ethanol a day for >10 years). With continued heavy alcohol consumption, alcoholic heart muscle disease progresses with the development of an alcoholic cardiomyopathy (for a review, see Refs. 52 and 66). Patients who develop alcoholic cardiomyopathy exhibit a worse clinical outcome compared with patients with a idopathic cardiomyopathy when alcohol consumption is not stopped or severely curtailed (49).

The etiology of the development of alcoholic cardiomyopathy is probably multifactorial. We (60, 61, 64, 66) and others (40, 41) have provided evidence that one potential mechanism to account for alcohol-induced alterations in ventricular function resides with a decrease in myofilibril and cellular proteins as a consequence of an inhibition of protein synthesis. Limiting mRNA translation causes the defect in protein synthesis. Altering the control of specific eukaryotic initiation (eIF) and elongation (eEF) factors appears to be the mechanism by which long-term ethanol ingestion reduces mRNA translation (22, 28, 61, 63, 64, 66).

All of the studies summarized above (22, 28, 61, 63, 64, 66) were performed in male animals. Gender-specific effects of chronic alcohol consumption on the myocardium are relatively unexplored. Lochner and colleagues (32) and Brown and colleagues (5) examined the effects of ethanol on contractile activity, whereas Piano (39) investigated changes in ventricular function. However, there are no studies examining the effects of sex differences on protein metabolism in the heart following chronic alcohol consumption. The purpose of the present set of experiments was to delineate sex differences in regulation of myocardial protein synthesis in response to long-term alcohol consumption.

MATERIALS AND METHODS

Chronic alcohol feeding. Pathogen-free, male and female Sprague-Dawley rats (Charles River Breeding Laboratories, Cambridge, MA) were maintained for 26 wk on an ethanol-containing diet, in which alcohol was provided both in drinking water and previously described agar blocks (4, 23, 55, 61, 64). Initially, age-matched rats (both male and female) were divided into two groups. In the control group, rats were maintained on a standard diet for 26 wk. In the ethanol group, rats were maintained on an ethanol-containing diet for 26 wk. The control diet consisted of laboratory chow and tap water. The ethanol-containing diet consisted of laboratory chow and a solution of ethanol in tap water. The ethanol solution was changed every day to ensure fresh ethanol. The alcohol concentration in the ethanol-containing diet was 5% (w/v). The rats were placed on a 12-h light-dark cycle, and the temperature and humidity were maintained at 22°C and 55%, respectively. The rats were housed in individually ventilated stainless-steel cages and were provided with food and water ad libitum. The rats were housed in individually ventilated stainless-steel cages and were provided with food and water ad libitum. The rats were housed in individually ventilated stainless-steel cages and were provided with food and water ad libitum. The rats were housed in individually ventilated stainless-steel cages and were provided with food and water ad libitum.
and female) were provided the agar block without ethanol for 2 days. The initial body weights in the males and females were 160 ± 2.9 (n = 30) and 133 ± 2.4 (n = 30), respectively. Thereafter, the male and female animals were randomly assigned to either an alcohol or control group. Animals in the alcohol group (males and females) were given free access to ethanol-containing agar blocks. The concentration of ethanol in the agar blocks was increased in 10% increments from 10% to 40% over the first 4 wk (22, 23, 28, 55, 61, 64). Ethanol-fed rats remained on the 40% ethanol-agar block diet for the remainder of the experimental protocol. Control agar blocks contained an equal caloric amount of dextrin-maltose. Standard rat chow (Harlan Teklad no. 8604, Madison, WI) provided nutrient intake in both groups. Male and female control rats and male rats consuming ethanol were provided the same amount of solid food as consumed by the female alcohol-fed group (22, 23, 28, 55, 61, 64). Total energy consumption was the same in both groups (26). Animals were weighed weekly, and during the course of the experiment, the volume of ethanol consumed was measured. Final body weights in the different groups were male-control: 602 ± 14 g; male-ethanol: 511 ± 9 g; female-control: 373 ± 11 g; female-ethanol: 340 ± 7 g.

Echocardiography. Echocardiograms were performed using the Sequoia C256 Echocardiography System (Siemens Medical Solutions, Mountain View, CA) equipped with a 7.5-MHz transducer 25 wk after initiating the ethanol-feeding regimen. Rats were anesthetized with ketamine (40 mg/kg) with acepromazine (1 mg/kg) intraperitoneally. Once anesthetized, animals were placed on a heating pad and covered with surgical towels to limit heat loss and maintain core body temperature during the procedure. The transducer was placed on the thorax, and M-mode recordings were performed by directing the ultrasound beam at the midpapillary muscle level. The measurements described herein were obtained after well-defined, continuous interfaces of the septal and posterior walls were visualized (18, 19, 31, 47). Measurements from three to four consecutive cardiac cycles were averaged for all animals. Intraobserver variability was less than 3% for the echocardiographic parameters measured (data not shown).

Protein synthesis. The rate of protein synthesis in vivo was determined using the flooding-dose technique described by Garlick et al. (15) and modified in our laboratory (55, 59, 61, 64, 65). Animals were anesthetized (Nembutal; 100 mg/kg body wt) and a polyethylene (PE)-50 catheter was surgically placed in the carotid artery. An initial 1 ml of blood was removed for measurement of blood ethanol concentrations. Subsequently, a bolus infusion of [3H]-L-phenylalanine (150 mM, 30 μCi/ml; 1 ml/100 gm body wt) was given via the jugular vein. At 2, 6, and 10 min after injection of the radioisotope, blood samples (1 ml) were drawn for measurement of phenylalanine concentrations and radioactivity. Immediately after the removal of the 10-min blood sample, the myocardium was excised, frozen between aluminum blocks, precooled to the temperature of liquid nitrogen. After which the frozen heart was weighed. Hearts were then powdered under liquid nitrogen with a mortar and pestle.

Approximately 0.2 g of powdered tissue was homogenized in 2 ml of ice-cold saline, and an aliquot was removed for measurement of total proteins using Bieрут method with crystalline serum albumin serving as a standard. Next, an equal volume of 3.6% (wt/vol) HClO4 was added. The sample was vortexed and subsequently centrifuged at 10,000 × g for 11 min at 4°C. The supernatant was decanted, and the pellet was washed with acetone, chloroform/methanol (1:1, vol/vol), and water. The pellet was dissolved in 0.1 M NaOH, and samples were assayed for protein. Another sample was assayed for radioactivity by liquid-scintillation spectrometry with corrections for quenching (disintegration per minute).

Rates of protein synthesis (%/day) were calculated as described earlier using the mean specific radioactivity of the plasma phenylalanine as the precursor pool (55, 59, 61, 64, 65). The specific radioactivity of the plasma phenylalanine was measured by HPLC analysis of supernatants from trichloroacetic acid extracts of plasma, as previously described (10). The specific radioactivities from the three time points were averaged. The specific radioactivity of the intracellular phenylalanine equals the specific radioactivity of the tRNA-bound phenylalanine under conditions of elevated plasma phenylalanine concentrations (>1 mM) (8, 33, 70).

Quantification of 4EBP1-eIF4E and eIF4G-eIF4E complexes by microtiter plate-based analysis. The association of eIF4E with 4EBP1 or eIF4G was determined in heart homogenates using a microtiter plate-based assay (20). Frozen powdered heart tissue was homogenized in 7 volumes of buffer A (in mM): 20 HEPES at pH 7.4, 100 KCl, 0.2 EDTA, 2 EGTA, 1 DTT, 50 NaF, 50 β-glycerophosphate, 0.1 PMSF, 1 benzamidine, 0.5 mM vanadate, and 1 μM microcystin LR, using a Polytron homogenizer. The homogenate was centrifuged at 10,000 × g for 10 min at 4°C, and the pellet was discarded. The homogenate was diluted with one-third volume of blocking solution (5% nonfat dry milk in phosphate-buffered saline). An aliquot was layered into wells of a microtiter plate previously coated with 1 μg of mouse monoclonal anti-eIF4E antibody (20, 37).

After incubation overnight, the sample was removed, the wells were washed, and, subsequently, either polyclonal anti-eIF4E (Cell Signaling Technology, Beverly, MA), anti-4EBP1, or anti-eIF4G (Bethyl Laboratories, Montgomery, TN) in blocking solution was added to the wells. The microtiter plate was incubated at room temperature for 1 h. Goat anti-rabbit minus mouse horseradish peroxidase-labeled antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) in blocking solution was added to wells. After four washes with PBS, TMB Super-Sensitive Microwell 1 Component Peroxidase Substrate (BioFX Laboratories, Owings Mills, MD) was added to the wells. Five minutes (for analysis of 4EBP1 content) or 30 min (for analysis of eIF4E or eIF4G content) later, STOP Reagent for TMB Microwell was added to each well. The absorbance at 450 nm was measured in an UVMap Kinetic Microplate Reader (Molecular Devices, Sunnyvale, CA). Nonspecific binding of eIF4E, eIF4G-eIF4E complex or 4EBP1-eIF4E complex is minimal. Values are expressed as the abundance of 4EBP1 or eIF4G divided by the abundance of eIF4E (20, 37).

Determination of phosphorylation state of 4EBP1. The phosphorylated forms of 4EBP1 were measured in heart extracts following boiling of an aliquot (200 μl) of the myocardial homogenates for 5 min. The boiled homogenate was centrifuged in a microcentrifuge at room temperature, and the supernatant was decanted. An equal volume of 2× Laemmli SDS buffer (65°C) was then added to the supernatant. The various phosphorylated forms of 4EBP1 (designated α, β, and γ) were separated by SDS-PAGE electrophoresis and quantitated by protein immunoblot analysis. The blots were then developed using an enhanced chemiluminesence (ECL) Western blot kit, as per the manufacturer’s instructions (Amersham Pharmacia Biotech, Piscataway, NJ). Films were scanned using a Microtek ScanMaker III scanner equipped with a transparent media adaptor connected to a Macintosh computer. Images were obtained using the ScanWizard Plugin (Microtek) for Adobe Photoshop and quantitated using NIH Image 1.63 software, as described previously (56, 58, 61).

Determination of phosphorylation state of eIF4G. To measure the relative extent of phosphorylation of eIF4G, an aliquot of the 10,000 g supernatant was mixed with an equal volume of 2× Laemmli SDS sample buffer (65°C) and then subjected to protein immunoblot analysis. Proteins were separated by 7.5% SDS-PAGE. After electrophoresis, the proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Biorad, PALL, Pensacola, FL). The membranes were incubated with antibodies specific for phosphorylated eIF4G (Ser1108) (Cell Signaling Technology, Beverly, MA) for overnight at 4°C. The blots were then developed as described above. After development of the immunoblot, the membranes were treated with a solution containing 62.5 mM Tris·HCl (pH 6.7), 100 mM β-mercaptoethanol, and 2% (wt/vol) SDS to remove antibodies, as per the manufacturer’s instructions. This procedure effectively removed all signal, resulting from...
Sex differences in the myocardial structure and function in animals consuming a diet containing ethanol measured using M-mode echocardiography.

### Table 1. Ethanol intake and blood alcohol concentrations in male and female rats

<table>
<thead>
<tr>
<th>Alcohol Consumption</th>
<th>Liquid Ethanol Intake, g·kg⁻¹·day⁻¹</th>
<th>Agar Block Ethanol Intake, g·kg⁻¹·day⁻¹</th>
<th>Total Ethanol Consumption, g·kg⁻¹·day⁻¹</th>
<th>Blood Alcohol Concentration, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>1.12±0.12</td>
<td>25.5±0.6</td>
<td>27±1</td>
<td>19±5</td>
</tr>
<tr>
<td>Female</td>
<td>4.27±0.74*</td>
<td>26.0±0.8</td>
<td>30±1*</td>
<td>24±5</td>
</tr>
</tbody>
</table>

Values shown are means ± SE. For alcohol consumption, the daily amount of the agar block and liquid ethanol solution consumed was measured daily and averaged over a two-week period. Rats were also weighed at the time of measurement of alcohol consumption. Because rats were housed in groups of two, the individual data points represent an average of each cage (n = 10). The blood alcohol concentration was measured at time of death (Males: n = 20, Females: n = 18). *P<0.001 vs. males.

### RESULTS

Ethanol intake and blood alcohol concentrations are shown in Table 1. During the final 4 wk of the experimental protocol, both male and female rats consumed approximately equal amounts of ethanol per day via the agar block (Table 1). In contrast, females drank 2.75-fold greater amounts of the 10% ethanol solution than males (Table 1). This observation is consistent with previous reports describing female rats show a preference for alcohol compared with their male counterparts when ethanol is presented as liquids (3, 7, 14, 21, 29). Hence, female rats consumed on average ~11% more ethanol than their male counterpart pairs (Table 1). Similar sex differences in ethanol consumption were observed in ethanol-preferring rats where the alcohol is presented in drinking water alone (50). Despite this difference in total ethanol consumption, the blood alcohol concentrations were not significantly different between the sexes, averaging 19±5 mM in males and 24±5 in females at the time of death. These ethanol values correspond with a blood alcohol content of ~0.1%, which is comparable to that observed in intoxicated humans displaying impaired mental and motor skills (69).

### In vivo changes in myocardial function.

We assessed the effects of sex and feeding rats a diet containing ethanol on the myocardial left ventricular stroke volume, heart rate, and cardiac output using M-mode echocardiography (Table 2). Prolonged feeding of male rats a diet containing ethanol resulted in a significant decrease in left ventricular stroke volume (~33%), and cardiac output (~11%) compared with pair-fed male control rats. In contrast to males, there were no significant changes in stroke volume or cardiac output in

### Table 2. Sex differences in the myocardial structure and function in animals consuming a diet containing ethanol measured using M-mode echocardiography

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Ethanol</th>
<th>Control</th>
<th>Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV systolic dimension, mm</td>
<td>2.0±0.2</td>
<td>1.50±0.08*</td>
<td>1.03±0.05†</td>
<td>1.11±0.04§</td>
</tr>
<tr>
<td>LV diastolic dimension, mm</td>
<td>7.1±0.3</td>
<td>6.7±0.2</td>
<td>0.61±0.2</td>
<td>6.2±0.1</td>
</tr>
<tr>
<td>LV Posterior Wall Thickness (LV PW), mm</td>
<td>2.36±0.05</td>
<td>2.02±0.06†</td>
<td>2.15±0.06*</td>
<td>2.07±0.05</td>
</tr>
<tr>
<td>IVS Dimension, mm</td>
<td>2.28±0.06</td>
<td>2.09±0.04*</td>
<td>2.02±0.04*</td>
<td>1.94±0.04§</td>
</tr>
<tr>
<td>IVS/LV PW</td>
<td>0.98±0.03</td>
<td>1.00±0.04</td>
<td>0.94±0.04</td>
<td>0.95±0.03</td>
</tr>
<tr>
<td>Left Ventricular Stroke Volume, ml</td>
<td>0.97±0.10</td>
<td>0.66±0.04†</td>
<td>0.53±0.003†</td>
<td>0.56±0.02</td>
</tr>
<tr>
<td>Heart Rate, beats/min</td>
<td>399±11</td>
<td>417±7</td>
<td>430±8</td>
<td>410±7</td>
</tr>
<tr>
<td>Cardiac Output, ml/min</td>
<td>320±36</td>
<td>284±18†</td>
<td>226±11†</td>
<td>223±8</td>
</tr>
</tbody>
</table>

Values shown are means ± SE for n = 9–24 in each group. Left ventricular Systolic Dimension (ANOVA P<0.0001; LV F = 11.64). LV posterior wall (LV PW) (ANOVA P<0.0005; F = 6.822). Intraventricular septal (IVS) (ANOVA P<0.0001; F = 8.965). IVS/LV PW (ANOVA P<0.05). Left ventricular stroke volume (ANOVA P<0.0001; F = 10.61). Cardiac output (ANOVA P<0.001; F = 10.68). *P<0.01, †P<0.001 vs. male control; §P<0.05 vs. male ethanol.
females fed a diet containing ethanol compared with pair-fed female control rats. However, stroke volume (−45%) and cardiac output (−30%) were significantly reduced in pair-fed control females compared with pair-fed male controls. The differences in these parameters between the sexes in controls are more than likely related to the smaller size of the female heart.

**Heart weight and myocardial protein content.** Chronic ingestion of alcohol significantly reduced the heart weight (left plus right ventricles) by 18% \((P < 0.01)\) in males (Table 3) compared with pair-fed male controls. The loss of heart weight was, in part, a result of a 14% drop in the amount of protein in the heart \((P < 0.001)\), such that the total amount of protein/heart was significantly decreased in male alcohol-treated rats compared with male controls (Table 3). In contrast, no differences in heart weight or myocardial protein content were observed between pair-fed control and alcohol-fed female rats (Table 3). The heart weight and protein content were significantly reduced in female control and female alcohol-fed animals compared with their male counterparts.

**In vivo measurement of myocardial structure.** Alterations in myocardial structure would be an expected consequence of the changes in ventricular mass and myocardial protein content. We assessed the effects of sex and feeding rats a diet containing ethanol on the myocardial left ventricular posterior wall thickness (LVPW) and interventricular septal (IVS) dimension using M-mode echocardiography (Table 2). Prolonged feeding of male rats a diet containing ethanol resulted in a significant decrease in both LVPW (−13%) and IVS (−9%) compared with pair-fed male control rats. The ratio of LVPW/IVS was not affected by ethanol consumption, indicating that there was no preferential reduction in the interventricular septum compared with posterior wall. In contrast to males, there were no significant changes in the LVPW and IVS in females fed a diet containing ethanol compared with pair-fed female control rats. However, the LVPW and IVS were significantly reduced in pair-fed control females (−9% and −12%) compared with pair-fed male controls.

**Rates of myocardial protein synthesis.** A significant 33% decrease was observed in the rate of protein synthesis in male ethanol-fed hearts compared with male control hearts (Fig. 1). The rate of protein synthesis in female hearts from control animals was significantly less (−34%) compared with their male counterparts. In contrast to males, there was no significant decrease in the rate of protein synthesis in hearts from female animals fed a diet containing alcohol compared with females not given ethanol.

**Regulation of eIF4E availability.** We investigated the effects of alcohol feeding on the association of eIF4G with eIF4E as a mechanism controlling mRNA translation. A 66% reduction in the amount of eIF4G associated with eIF4E was observed in hearts from males fed a diet containing ethanol compared with male controls (Fig. 2). The amount of eIF4G associated with eIF4E in female hearts from control animals was significantly less (−50%) compared with male control hearts. In contrast to males, there was no significant additional decrease in the assembly of eIF4G·eIF4E in hearts from female animals fed a diet containing alcohol compared with female control hearts.

The availability of eIF4E for binding to eIF4G can be regulated by its association with the translation repressor, 4EBP1. A significant twofold increase in the total amount of 4EBP1 associated with eIF4E was observed between male ethanol-fed hearts compared with male control hearts (Fig. 3). The amount of 4EBP1 associated with eIF4E was greater (+36%) in hearts from pair-fed control females, compared

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**Table 3. Sex differences in the myocardial mass and protein content in animals consuming a diet containing ethanol**

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Ethanol</td>
</tr>
<tr>
<td>Heart weight, gm</td>
<td>1.7±0.09</td>
<td>1.4±0.03§</td>
</tr>
<tr>
<td>Total myocardial protein, mg/heart</td>
<td>316±19</td>
<td>272±6†</td>
</tr>
</tbody>
</table>

Values shown are means ± SE for \(n = 8–19\) in each group. Heart weight (ANOVA \(P < 0.0001; F = 27.512\)). Myocardial protein (ANOVA \(P < 0.0001; F = 29.72\)) \(*P < 0.01; †P < 0.001\) vs. male control; ‡\(P < 0.001\) vs. male ethanol.
with pair-fed control male rats. There was no significant additional increase in the assembly of 4EBP1/eIF4E in hearts from female animals fed a diet containing alcohol compared with females not fed ethanol.

The interaction between 4EBP1 and eIF4E appears regulated by the extent of 4EBP1 phosphorylation (30, 38). Because of the differences in the association of eIF4E with 4EBP1 between control and alcohol-consuming rats and between the sexes, we examined potential changes in 4EBP1 phosphorylation. The total myocardial content of 4EBP1 (sum of all three phosphorylated forms) did not differ significantly between control (11,539 ± 1,393 arbitrary units/mg protein) and alcohol-treated (12,028 ± 1,206 arbitrary units/mg protein) rats. The amount of 4EBP1 in the γ-form in cardiac muscle of male rats fed a diet containing ethanol was decreased ~25% compared with male control animals (Fig. 4). Likewise, the amount of 4EBP1 in the γ-form in cardiac muscle of female rats fed a diet containing ethanol was less (~14%) compared with male control animals. There were no significant differences in the phosphorylation of 4EBP1 in hearts from female animals fed a diet containing alcohol compared with control females. However, there was an increase in the phosphorylation of 4EBP1 in hearts from female animals compared with males fed a diet containing alcohol.

Phosphorylation of eIF4G. The interaction between eIF4E and eIF4G can be regulated, at least in part, by phosphorylation of eIF4G on Ser1108 in addition to availability of eIF4E. We did not observe any significant differences in the phosphorylation of eIF4G in male or female animals fed a diet containing ethanol [male: 70 ± 2 arbitrary units (AU); female: 70 ± 2 AU] compared with pair-fed controls (male: 68 ± 4 AU; female: 71 ± 3 AU). Therefore, it does not appear that the phosphorylation of eIF4E is important in controlling mRNA translation initiation in hearts from animals fed a diet containing ethanol. Furthermore, there are no sex differences in response to ethanol.

Expression of contractile proteins. The effect of feeding male and female rats a diet containing alcohol on the myocardial content of contractile proteins is shown in Fig. 6. The relative amount of α-myosin heavy-chain isoform and actin was reduced by ~45% and ~38% in hearts from male ethanol-fed rats compared with male pair-fed controls (Fig. 6). In contrast, the relative abundance of β-myosin heavy chain males, feeding a diet containing alcohol significantly reduced the extent of phosphorylation of eIF4G in cardiac muscle (Fig. 5). In contrast, no significant differences were observed in the extent of phosphorylation of eIF4G between female control and alcohol-fed female rats.

Phosphorylation of eIF4E. Several reports suggest that phosphorylation of eIF4E may enhance binding of mRNA to eIF4E (42, 44, 45, 48). We did not observe any significant differences in the phosphorylation of eIF4E in male or female animals fed a diet containing ethanol [male: 70 ± 2 arbitrary units (AU); female: 70 ± 2 AU] compared with pair-fed controls (male: 68 ± 4 AU; female: 71 ± 3 AU). Therefore, it does not appear that the phosphorylation of eIF4E is important in controlling mRNA translation initiation in hearts from animals fed a diet containing ethanol. Furthermore, there are no sex differences in response to ethanol.

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isoform was greater (+143%) in hearts from male ethanol-fed rats compared with male pair-fed controls (Fig. 6).

In contrast to males, there was a significant increase in the expression of α-myosin in hearts from female ethanol-fed rats compared with pair-fed female controls (Fig. 6). Furthermore, there were no significant changes in the expression of either actin or β-myosin in hearts from female ethanol-fed rats compared with pair-fed female controls (Fig. 6).

DISCUSSION

Diseases related to excessive long-term alcohol abuse afflict both men and women. Among all patients presenting with alcoholic cardiomyopathy, men represent the largest percentage, and death rates are higher in men than in women. The prevailing general clinical impression suggests that few female alcoholics develop alcoholic cardiomyopathy, even adjusting for the lower incidence of alcoholism in women. Despite these observations, the cardiotoxicity of ethanol does not spare the female heart (51). Women alcoholics exhibit features associated with alcoholic heart muscle disease with symptoms consistent with heart failure. The majority of women diagnosed with alcoholic cardiomyopathy are classified in New York Heart Association (NYHA) functional class II, whereas the majority of men are classified as being associated with NYHA class III or IV (11).

However, the molecular mechanisms responsible for differences between men and women exposed to chronic ethanol consumption remain unknown.

Alcoholic heart muscle disease appears more pronounced in male than female rats (17) and is characterized histologically by a thinning of the ventricular walls and loss of myofibrils (1, 2, 6, 12). In the present study, prolonged alcohol consumption caused a decrease in both IVS and posterior wall thickness in males, consistent with the reports in humans. The ratio of IVS/LVPW was unaffected by chronic ethanol consumption, providing no evidence of a preferential loss of left ventricular myocardium. The reductions in left ventricular wall thickness produced by chronic alcohol exposure were accompanied by a corresponding decrease in the heart weight. In contrast, hearts from female rats were not affected by feeding animals a diet containing ethanol. These distinctions between males and females were not the result of disparities in the blood alcohol...
concentrations, even though females consumed more ethanol. This observation may relate to increased elimination of ethanol in females during first pass through the liver through potential changes in enzymes that metabolize ethanol. Hence, sex differences in the structure of the myocardium exist in the response to chronic ethanol feeding.

The mechanism responsible for a decreased left ventricular mass may relate to reductions in the protein content of the heart. In the present set of experiments, myocardial protein content was decreased in hearts from male rats fed a diet containing ethanol. As was observed with the heart weight and ventricular wall thickness, no significant alteration in the protein content in response to ethanol was observed in hearts from female rats. Reduced organ protein content would be expected to alter the normal functioning of the myocardium through a lowering in the abundance of enzymes and proteins necessary to maintain cellular homeostasis and function. Indeed, male ethanol-fed rats had a lower stroke volume and cardiac output compared with pair-fed male controls.

The dynamic balance between rates of protein synthesis and degradation regulates cellular protein content. Rates of protein synthesis remained lower in hearts from male animals fed a diet containing ethanol for 26 wk compared with male pair-fed controls. In contrast, rates of protein synthesis in female rats consuming ethanol were not reduced compared with female pair-fed controls. This reduction in protein synthesis in males occurred despite equal caloric and nitrogen intake between the pair-fed control and alcohol-fed groups. Hence, the derangements in protein synthesis over the course of the 26-wk feeding regime appear independent of nutritional status of the alcohol-fed rats.

The potential mechanisms responsible for the inhibition of protein synthesis in hearts from males consuming a diet containing ethanol are beginning to be elucidated. Ethanol limits protein synthesis at the step involved in the binding of mRNA to the 43S preinitiation complex. Indeed, a decreased formation of an active eIF4E/eIF4G complex may limit mRNA translation initiation in intoxicated male rats (24, 27, 61). In the present study, reduced assembly of eIF4E/eIF4G complex following 26 wk of chronic ethanol administration would be expected to diminish the association of mRNA with the ribosome and hence limit protein synthesis in male rats. In contrast, no significant reduction in the assembly of eIF4E/eIF4G complex was seen in hearts from females rats fed a diet containing ethanol compared with pair-fed control females. This latter observation is consistent with the role of an active eIF4G/eIF4E complex in regulating protein synthesis, as ethanol failed to reduce rates of protein synthesis in hearts from female rats compared with males consuming a diet containing alcohol.

One potential mechanism for decreased binding of eIF4E to eIF4G in males consuming ethanol may involve phosphorylation of eIF4G (36, 43). Increased phosphorylation of eIF4G correlates with conditions known to stimulate protein synthesis (35, 43). Currently, there is no information available concerning the effect of chronic alcohol intoxication on the phosphorylation of eIF4G in cardiac muscle. The extent of phosphorylation of eIF4G was significantly reduced (50%) in hearts from male ethanol-fed rats compared with pair-fed male controls. The decreased phosphorylation of eIF4G in males consuming alcohol is not the result of alterations in the myocardial

Fig. 6. Effect of chronic alcohol feeding on relative amount of α-myosin heavy chain isoform, β-myosin heavy chain isoform, and actin. Equal amounts of protein derived from homogenates from the hearts described in Fig. 1 were electrophoresed, transferred to polyvinylidifluoride membranes and analyzed by Western blot for myosin isoforms and actin. Representative immunoblots and densitometric analysis of several immunoblots are shown for α- and β-myosin heavy-chain isoforms and actin. Values shown are means ± SE for 7–16 animals in each group. *P < 0.05 vs. male control. †P < 0.01 vs. male control. §§ P < 0.05 vs. female control. ‡P < 0.001 vs. male EtOH (α-myosin ANOVA P < 0.001, F = 8.42; actin ANOVA P < 0.001, F = 6.83, β-myosin ANOVA P < 0.001, F = 12.8).
content of eIF4G, as the cellular content of eIF4G is not different in animals injected with alcohol compared with controls (data not shown). This finding is in contrast to enhanced myocardial eIF4G phosphorylation observed after acute ethanol intoxication (binge drinking) (54). Hence, phosphorylation of eIF4G decreases in males with increasing duration of ethanol consumption.

If phosphorylation of eIF4G is an important regulator of protein synthesis in cardiac muscle, phosphorylation of eIF4G should not decrease in female ethanol-fed rats compared with their pair-fed controls because assembly of eIF4G-eIF4E is unaffected by chronic ethanol feeding. Indeed, this is precisely what we observed. Hence, diminished phosphorylation of eIF4G appears important in causing the reduced formation of active eIF4E-eIF4G complex in hearts from male animals fed a diet containing ethanol.

However, other mechanisms may potentially modulate the extent of assembly of eIF4G-eIF4E in hearts from male animals fed a diet containing ethanol. Assembly of eIF4G-eIF4E can also be modulated through availability of eIF4E through the association of eIF4E with a family of small, acid- and heat-stable proteins, termed 4EBP1, 4EBP2, and 4EBP3 (16). 4EBP1 is the predominant form of these translation repressor proteins in cardiac muscle (Vary TC and Kimball SR, unpublished results). 4EBP1 binding to eIF4E prevents the association of eIF4G with eIF4E and essentially limits cap-dependent mRNA translation by physically sequestering eIF4E into an inactive complex, thereby limiting binding of mRNA to the ribosome (16). In the present set of experiments, feeding male rats a diet containing ethanol caused a ~16% increase in the amount of 4EBP1 associated with eIF4E in cardiac muscle. In contrast, no significant differences in the amount of 4EBP1 associated with eIF4E were detected in cardiac muscle from female rats compared with pair-fed female controls. In addition to decreases in phosphorylation of eIF4G, increased binding of eIF4E to 4EBP1 appears important in limiting the assembly of eIF4G-eIF4E in hearts from male rats fed a diet containing ethanol.

Binding of 4EBP1 to eIF4E is controlled by its phosphorylation state. The most highly phosphorylated form of the protein, 4EBP1γ, does not bind eIF4E. Reductions in the amount of 4EBP1γ in alcohol-fed male rats would be expected to increase the binding of eIF4E to 4EBP1. Such a scenario was observed in hearts from males rats where feeding rats a diet containing ethanol diminished phosphorylation of 4EBP1γ by ~25%. In contrast, no decreases in phosphorylation state of 4EBP1γ were observed in cardiac muscle from females following ethanol feeding. Hence, the alcohol-induced increase in eIF4E-4EBP1 complex in male rats most likely occurred through a reduced phosphorylation of 4EBP1γ.

Changes in regulation of eIF4G-eIF4E assembly would be expected to modify the mRNA translation and thereby lower rates of protein synthesis. In this regard, we have previously established that translational control of mRNA expression plays an important role in the regulation of actin, α-myosin, and β-myosin expression after 16 wk of chronic ethanol consumption (55). In the present set of experiments, similar changes in actin (~38%), α-myosin (~45%), and β-myosin heavy-chain isofrom (~143%) were observed in hearts from male animals fed a diet containing ethanol for a longer period of ethanol feeding. These observations are consistent with previous reports showing the ratio of β- to α-myosin heavy-chain isoforms increases dramatically in hearts from male rats fed a diet containing ethanol (13, 34, 55).

Compared with males, the abundance of myosin and actin was significantly less in hearts from control females compared with male controls. This finding is also consistent with translational control of mRNA expression of structural and functional proteins in hearts from female rats. This is evidenced by a higher mRNA expression of α- and β-myosin heavy chain and actin in female adult hearts (46) coupled with a lower eIF4G-eIF4E complex. Moreover, a different pattern of myofibrillar protein expression was observed in hearts from female rats fed a diet containing ethanol. Chronic ethanol consumption caused a significant increase in the expression of α-myosin in hearts from female rats compared with pair-fed female controls. Furthermore, neither actin nor β-myosin expression was altered in hearts from female rats fed a diet containing ethanol compared with pair-fed controls. Therefore sex differences exist in the response of myocardial protein expression to chronic ethanol consumption.

In summary, the studies presented herein provide evidence for sex differences in the myocardial response to chronic alcohol consumption at the molecular level. In control animals, hearts from female rats showed lowered heart weights and had thinner ventricular walls compared with males. The smaller heart size was associated with a lower amount of protein that occurred, in part, from a reduced rate of protein synthesis. Chronic alcohol consumption in males caused a thinning of the ventricular wall and intraventricular septum, which was associated with a loss of heart mass; an effect not found in females consuming ethanol. The alterations in cardiac size occurred in part through a lowering of the protein content secondary to a diminished rate of protein synthesis. The decreased rate of protein synthesis appeared related to a reduced assembly of active eIF4G-eIF4E complex secondary to both a diminished phosphorylation of eIF4G and increased formation of inactive 4EBP1-eIF4E complex. The latter effects occurred as a result of decreased phosphorylation of 4EBP1. The upstream regulatory protein mTOR is a key factor in controlling the phosphorylation of components involved in translation initiation under a variety of conditions. However, it remains to be established whether the differences in the degree of eIF4G-eIF4E association and/or eIF4G phosphorylation result from changes in mTOR activity in response to chronic alcohol consumption. None of these ethanol-induced alterations in hearts from males were observed in hearts from females. Therefore, females appear less affected by long-term alcohol consumption than their male counterparts over the time frame examined. This is at least consistent with the human studies. However, our results do not preclude the possibility that with longer periods of exposure or greater alcohol consumption, females would show deleterious effects of ethanol. These data suggest that chronic alcohol-induced impairments in myocardial protein synthesis results, in part, from marked decreases in eIF4E-eIF4G complex formation in males. The failure of female rats consuming ethanol to show structural changes appears related to the inability of ethanol to affect the regulation of protein synthesis to the same extent as their male counterparts.
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