Functional proteomic analysis reveals sex-dependent differences in structural and energy-producing myocardial proteins in rat model of alcoholic cardiomyopathy

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Alcohol abuse remains one of the most common forms of drug abuse among both sexes in the United States. Although the risk of cardiovascular diseases is reduced with moderate alcohol consumption, potential consequences of excessive and chronic alcohol abuse, defined as >80 g of alcohol per day for longer than 5 yr, include heart dysfunction and heart failure (47). This quantity and frequency of ethanol consumption are sufficient to induce alcoholic heart muscle disease (AHMD) and, in approximately one-third of the cases, contribute to the development of a dilated cardiomyopathy (DCM: 48, 50). AHMD is rarely the result of short-term ethanol intake but is more commonly observed in patients who consume excessive amounts of alcohol for prolonged periods (48, 50). This disease is clinically characterized by adverse alterations in both the structure and function of the myocardium, including ventricular dilation, thinning of the ventricular wall, and myocardial dysfunction (17, 30, 32, 43). The degree of cardiac dysfunction is proportional to the duration and severity of alcohol consumption (38). The functional and structural changes observed with the progression of the disease to a dilated heart are consistent with alterations in steps in protein synthesis inhibition that have been implicated in messenger RNA selection for translation (10, 20, 41, 45). Therefore, it seems reasonable that the expression of specific proteins may be affected. However, the alcohol-induced changes in ventricular protein levels that initiate the progression from a healthy, functioning heart to one exemplified by DCM are currently not understood.

The differential effects of the pathogenesis of AHMD across the sexes have also not been extensively studied. Although the effects of chronic alcohol intake are frequently studied in males, females are also susceptible to alcohol-induced myocardial defects. Sexually dimorphic responses to alcohol have been observed in clinical studies (11, 38, 49) and animal models (6, 24, 32, 43) in terms of the occurrence, manifestation, and outcome of AHMD. Specifically, the myocardial mass and protein content are decreased in males, but not females, as a result of consuming an alcohol-containing diet (32, 43). Furthermore, a shift from alpha-to-beta myosin, which is often observed in disease states (18), is evident in male rats but not female rats (43). While this model of AHMD suggests that female rats are protected from the toxic effects of alcohol, other studies report that female rats do in fact develop a DCM although not to the same extent as male rats (32). Furthermore, to date, only selected candidate proteins have been investigated (8, 40) and, therefore, the full extent to which myocardial proteins are affected by chronic alcohol consumption across the sexes remains unresolved. Given the amount of contradictory data pertaining to the sex-dependent effects of long-term ethanol consumption on the myocardium, a better understanding of the pathophysiology of AHMD between male and female rats seems warranted.

Therefore in this study we have used proteomic approaches to examine expression of proteins in hearts from male and female rats after chronic alcohol exposure. Our model represents an early stage of AHMD, with the distinct advantage of allowing identification of protein content alterations that precede end-stage DCM. To increase the number of proteins...
identified we examined different centrifugal fractions and developed a statistical approach to combine and determine statistical differences from data obtained from different experiments or assays. The relevance of sexually dimorphic changes observed in myofibrillar proteins essential to contraction and mitochondrial proteins involved in oxidative phosphorylation in the dimorphic response to AHMD are discussed.

METHODS

Experimental design: chronic alcohol feeding. All experimental protocols involving animals were approved by the Institutional Animal Care and Use Committee of The Pennsylvania State University College of Medicine and adhered to the National Institutes of Health guidelines for the use of experimental animals. Male and female Sprague-Dawley rats (Charles River Breeding Laboratories, Cambridge, MA) were maintained on an alcohol-containing diet for 18 wk with housing and pair feeding as previously described (12, 22, 41). All rats were acclimated to receiving alcohol-free flavored agar block for 6 days. The initial body weights in males and females were 152.7 ± 12.7 (n = 10) and 131.9 ± 11.0 (n = 10), respectively. Thereafter, the male and female animals were randomly assigned to either Alcohol or Control group, resulting in four study groups (female control, male control, female alcohol, male alcohol). The Alcohol group was provided ethanol via drinking water (10% vol/vol) and previously described flavored agar blocks (4, 40, 41, 44, 45). The concentration of ethanol in the agar blocks was increased 10% per week from 10 to 40% over the first 4 wk and thereafter was maintained at 40%. Animals in the Control group were provided ethanol-free agar blocks containing an isocaloric amount of dextrin-maltose to balance the calories present in the ethanol-containing blocks of the Alcohol group. Agar consumption was measured weekly. All groups were also fed a diet to generate two fractions according to procedures used previously in our laboratory (42) and originally adapted from Fuller and Randle (13). The heart was minced with scissors before being transferred to a blender (Waring commercial blender 7012, model 34BL97; Waring Products Division - Dynamics of America, New Hartford, CT) containing 180 ml of ice-cold isolation buffer (100 mM Tris-HCl, pH 7.4; 200 mM mannitol, 70 mM sucrose, 10 mM EDTA). Each sample was blended for 12.5 s. The mixture was diluted further by an additional 100 ml of the ice-cold 100 mM Tris-HCl isolation buffer and blended again for 12.5 s. Contents were poured into a glass beaker on ice containing 50 mg trypsin/20 ml of isolation buffer and stirred for 12.5 min. Contents were then filtered through a fine sieve (woven nylon mesh, 0.710 mm sieve opening) into 300 ml bottles and centrifuged at 540 g at 4°C for 12 min. The pellet (fraction 1) was resuspended and saved. The supernatant was filtered through a fine sieve into different 300 ml bottles and centrifuged at 12,170 g at 4°C for 12 min. The supernatant was discarded while the pellet (fraction 2) was suspended in 10 mM Tris-HCl isolation buffer. The resuspended pellet was then centrifuged in one 30 ml tube at 9,475 g at 4°C for 12 min, and the resulting pellet (fraction 3) was suspended in 10 mM Tris-HCl isolation buffer and saved.

To ensure that equal protein amounts were used for the generation of two additional fractions according to procedures used previously in our laboratory (40, 46) and originally adapted from Hoover-Plow and Clifford (14). Cardiac muscle samples were homogenized using a motor-driven glass-on-glass tissue grinder (Thomas Scientific, Swedesboro, NJ) and recentrifuged in 30 ml bottles at 9,475 g at 4°C for 12 min. Supernatant was discarded and pellet resuspended in 10 mM Tris-HCl isolation buffer. The resuspended pellet was then centrifuged in one 30 ml tube at 9,475 g at 4°C for 12 min, and the resulting pellet (fraction 2) was dissolved in 1–2 ml of 10 mM Tris-HCl isolation buffer and saved. For this protocol, it was necessary to combine ventricular sample from two different animals, of the same sex (male or female) and of the same group (alcohol or control), to have enough protein for subsequent analyses. As a result of pooling samples, this protocol resulted in n = 3 for fraction 1 and n = 3 for fraction 2 for each of the four study groups.

The remaining half of the perfused heart (~0.5 g) was used for the generation of two additional fractions according to procedures used previously in our laboratory (40, 46) and originally adapted from Hoover-Plow and Clifford (14). Cardiac muscle samples were homogenized using a motor-driven glass-on-glass homogenizer in 7.5× volume of ice-cold 10 mM KH2PO4 (pH 7.4) buffer. The samples were centrifuged at 2,485 g at 4°C for 20 min. The supernatant (fraction 3) was saved for subsequent analyses. The pellet (fraction 4) was washed with 2 ml of 0.1 M KH2PO4 (pH 7.4) buffer and then centrifuged at 2,485 g at 4°C for 20 min. The resulting supernatant was removed and saved as part of fraction 3. The pellet was washed a second time, and the two supernatants were pooled (fraction 3). The pellet (fraction 4) was suspended in 2 ml of 0.6 M NaCl, centrifuged at 550 g at 4°C for 60 s, and the supernatant was saved as fraction 4. This protocol resulted in n = 5 for fraction 3 and n = 5 for fraction 4 for each of the four study groups.

Quantitation of protein. To ensure that equal protein amounts were subjected to proteomic analysis, protein concentrations of the centrifuged fractions were first determined via bicinchoninic acid (BCA) protein assay using bovine serum albumin (BSA) as a standard according to the manufacturer’s directions (Pierce, Rockford, IL). In addition, aliquots of the centrifuged fractions were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and silver stained according to the manufacturer’s instructions for conventional slab gels (GE Healthcare, Piscataway, NJ). Gels were dried in an Easy Breeze Gel Drying System (Hoefer Scientific Instruments, Holliston, MA). Dried gels were scanned (Epson Perfection 4990 Photo; Epson, Long Beach, CA), and the staining intensity of the
protein pattern was compared by densitometric analysis (Image J Software).

Western blot analysis. Samples were mixed with either an equal volume of 2× Laemmli SDS sample buffer or one-fourth the volume of 5× sample buffer (60°C). The samples were mixed, boiled for 3 min, and briefly centrifuged. Equal amounts of protein were subjected to SDS-PAGE using either 12.5 or 15% acrylamide gel followed by transfer of proteins to polyvinylidene fluoride (PVDF) membranes (Pall, Pensacola, FL), as described previously (21, 41). After being blocked with resuspended dried milk, the membranes were incubated with antibodies specific for aconitase 2, troponin T, troponin C, or troponin I and subsequently developed. Commercially available antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, troponin I and subsequently developed. Commercially available antibodies specific for aconitase 2, troponin T, troponin C, or troponin I).

iTRAQ application and sample preparation. Samples were prepared according to the Pennsylvania State University College of Medicine Mass Spectrometry and Proteomics Core Facility standard protocol (http://www.hmc.psu.edu/core/proteins_MassSpec/MassSpec/iTRAQ%20Protocol.htm), adapted from the manufacturer’s instructions (Applied Biosystems, Carlsbad, CA). A total of five samples per study group were prepared for fractions 3 and 4, while three samples per study group were prepared for fractions 1 and 2. Protein (150 μg per sample) from the centrifuged fractions were brought to the same starting volume using the sample preparation buffer, snap-frozen in liquid nitrogen, and lyophilized. Each sample was initially dissolved in 20 μl of 0.5 M triethylammonium bicarbonate (pH 8.5). Samples were then denatured and reduced by bringing the samples to a final concentration of 0.01% SDS and 5 mM tris-(2-carboxyethyl) phosphine and then incubating them at 60°C for 1 h. Samples were brought to a concentration of 3.65 mM iodoacetamide and incubated for 30 min at room temperature while protected from light. Samples were then digested with 5 μl of sequencing grade trypsin (Promega) at 48°C overnight (12–16 h). A portion (40–50 μg) of each sample was subjected to SDS-PAGE using 15% acrylamide gel and subsequent silver staining to verify the trypsin digestion.

Four-plex iTRAQ technology was utilized to selectively analyze a single centrifuged fraction across each of the four study groups. One tube of each iTRAQ reagent (114, 115, 116 or 117) was dissolved in 70 μl of ethanol. The remainder of the digested samples (100 μg) were labeled with the appropriate iTRAQ reagent, incubated for 1 h, and quenched by addition of 100 μl of water. All of the individual samples, representing each of the four study groups and now labeled with iTRAQ reagent, were then combined. The combined sample was then snap-frozen in liquid nitrogen prior to being subjected to three rounds of drying in a SpeedVac, which used a lyophilizer as a cold trap, and resuspension with 200 μl of water. Following the third round of drying, 500 μl of 12 mM ammonium formate made in 25% acetonitrile (pH 2.5–3.0), was added to the combined sample prior to two-dimensional liquid chromatography separation, mass spectrometry analysis, and subsequent protein identification and quantitation. Each subsequent iTRAQ application was completed on an independent sample (i.e., no technical replicates), and therefore, when multiple iTRAQ experiments were completed with sample obtained from a particular centrifuged fraction these represented biological replicates.

The iTRAQ-labeled peptides were resolved by two-dimensional liquid chromatography prior to matrix-assisted laser desorption and ionization (MALDI) time-of-flight (TOF) tandem mass spectrometry (MS/MS) as described previously (16, 51). Briefly, the samples were separated into 15 strong cation exchange (SCX) fractions, each of which was separated on an LC-Tempo nanoflow and MALDI spotting system, resulting in a total of 370 spots per SCX fraction. MALDI target plates were analyzed in a data-dependent manner on an ABI 4800 MALDI TOF-TOF. MS/MS was performed with collision-induced dissociation of selected peaks.

Peptide identification, protein grouping, and subsequent protein quantitation were done using the Paragon and ProGroup Algorithms (35) in the ProteinPilot Software package (ABSciex, Foster City, CA), searching the NCBI-h Rat database plus a list of 170 common contaminants, concatenated with a decoy database containing the reversed sequences of the original, normal database. This search of a concatenated normal and reversed database allowed estimation of the false discovery rate (FDR) using the PSPEP algorithm (37) so that a cutoff was created where identifications are only accepted when the local FDR of the lowest ranking protein is <5%. Therefore, all higher-ranking proteins will have decreasingly lower probabilities of being false positives.

In summary, our data consisted of four study groups: female control (FC), male control (MC), female alcohol (FA), and male alcohol (MA). In addition, for each of these study groups there were four different centrifuged fractions, designated as fraction 1, fraction 2, fraction 3, and fraction 4. As a result of these numbers, multiple 4-plex iTRAQ experiments were necessary. A total of five iTRAQ experiments were performed for fraction 3 and fraction 4, while three iTRAQ experiments were performed for fraction 1 and fraction 2, as detailed above. The difference in the number of iTRAQ experiments performed among the fractions was a result of two hearts being combined to have enough protein for the one centrifugation protocol. The four-plex iTRAQ technology allowed simultaneous analysis of a centrifuged fraction across each of the four study groups. Within each iTRAQ experiment there were several comparisons of interest, for example, the effect of alcohol (FA:FC and MA:MC) or inherent differences between the sexes (FC:MC and FA:MA).

Statistical analysis. To determine whether protein ratios differ significantly across subjects, the ratios were modeled using a random effects model. The random effects model assumed that individual iTRAQ experiments estimate different protein ratios and that these different ratios were being drawn from a larger distribution with some mean and standard deviation. Formally, each protein ratio from an individual iTRAQ experiment was assumed to have normal distribution, with a mean ρ. The underlying statistical model of the (natural log) protein ratio is $R_i = \theta_i + \epsilon_i$, where $i$ indexes an iTRAQ experiment, $R_i$ is a (log) protein ratio from experiment $i$, $\theta_i$ is a location parameter (e.g., mean for a normal distribution), and $\epsilon_i \sim N(0, \sigma^2)$ is a zero-mean, normally distributed error term. In addition, the ratio is modeled as being drawn from a larger distribution so that $\theta_i = R + \delta$, where $\delta \sim N(0, \mu^2)$ is also a zero-mean, normally distributed error term. $R$ represents the overall protein ratio (across all animals and iTRAQ experiments) and is the final object of inference. An estimate of $R$ gives an estimate of the overall ratio across iTRAQ experiments and the confidence interval of $R$ gives a measure of uncertainty in the estimate. The null hypothesis is that $R = 1$, which implies the same protein concentration in each group. By substitution, the model becomes $R_i = (R + \mu) + \epsilon_i$ and with further substitution, $R_i = R + \delta$, where $\delta_i = \epsilon_i + \mu$ is a zero-mean, normally distributed error term that captures both the within-experiment variation ($\mu$) and the between-experiment variation ($\epsilon_i$). With this formulation of the model, the overall protein ratio that can be obtained as an weighted average of individual ratios from each iTRAQ experiment

$$R^* = \frac{\sum_{i=1}^{n} w_i f_i}{\sum_{i=1}^{n} w_i}$$

where the weight for experiment $i$ is defined as $w_i = (\sigma^2 + \tau^2)^{-1}$. In other words, the weight for a protein identification within an iTRAQ experiment is proportional to the inverse of the variance (i.e., the normally distributed error term associated with each identification). As a result, ratios that are more precisely estimated, or have less random variation, are given greater weight in the pooled estimate. The
overall protein ratio was considered statistically significant at the 5% level if the 95% confidence interval did not contain 1.

An identifier (i.e., experiment = 1, . . . , 5) was created for each experiment to distinguish which proteins were identified in which experiment. All datasets for a particular centrifugal fraction were then merged and saved as one large dataset, containing the run identifier and the variance of that particular identification. For proteins identified in a single dataset, a t-test utilizing the ratio and error factor allowed determination of statistically significant proteins. With this approach, a single-identification protein with a statistically significant ratio was considered as a potential protein of interest. Proteins identified in multiple iTRAQ experiments were required to have a statistically significant ratio after meta-analysis to be considered a protein of interest.

Algorithms for combining proteins were programmed using STATA (version MP 9.2 for Macintosh) and R (version 2.8.1, http://www.r-project.org), an open-source environment for statistical computing and graphics, and the rmeta package (version 2.15).

RESULTS

Experimental design: chronic alcohol feeding. Body weights were measured weekly during the alcohol exposure (Fig. 1A) along with final body weights at the time of tissue removal (Fig. 1B). No difference in body weights was observed between alcohol and the respective control groups, although both female groups weighed less than males, as expected (P < 0.001). Throughout the experimental protocol, alcohol consumption did not differ between male and female rats when intake was normalized to body weight (Fig. 2).

Echocardiographic parameters. Chronic ingestion of alcohol reduced left ventricular heart weight by 14% (P < 0.05) in males compared with pair-fed male controls (Table 1, LV mass). In contrast, no difference in left ventricular weight was observed between pair-fed control and alcohol-fed female rats. As expected, left ventricular weights in female control animals were significantly lower compared with their control male counterparts.

The effects of sex and ethanol on functional echocardiographic endpoints, including stroke volume (SV) and cardiac output (CO), were assessed via noninvasive M-mode echocardiographic evaluation (Table 1). Chronic ethanol significantly decreased SV (−33%) and CO (−28%) in male rats compared with pair-fed controls. In contrast to males, there was no significant change observed in either SV or CO in female rats fed a diet containing ethanol compared with pair-fed female control rats.

Alterations in myocardial structure would be an expected consequence of the changes in ventricular mass and measurements of myocardial function. We assessed the effects of sex and ethanol exposure on structural parameters, including the dimension of the left ventricle during both diastole and systole (EDD and ESD), LV PW thickness and IVS dimension, using M-mode echocardiography (Table 1). Males consuming alcohol displayed a significantly reduced EDD (−10%) compared with their respective control group. In contrast to males, however, there was no significant change observed in EDD in female rats fed a diet containing ethanol compared with pair-fed female control rats. Females in both control and alcohol-fed female rats normalized to body weight. Ethanol intake in male and female rats was normalized to body weight for the final 5 wk of the alcohol-containing diet. Values are shown as means ± SE. The amount of agar block consumed was measured weekly to determine ethanol consumption. Because rats were housed in pairs, the data represent an average of each cage. Rats were also weighed at the same time alcohol consumption was measured. As females weighed significantly less than males, the amount of ethanol intake was normalized to kg body weight to determine if there was a difference in the level of consumption.

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groups demonstrated a decrease in EDD when matched with the respective male group. No change was observed in ESD for either males or females consuming alcohol compared with the respective control groups. Furthermore, the ratio of IVS/LVPW was not affected by ethanol consumption, indicating that there was no preferential reduction in either IVS or LVPW measurements in relation to one another.

**iTRAQ analysis: identifying proteins of interest.** Three 4-plex iTRAQ experiments were completed using fractions 1 and fractions 2, whereas samples obtained from fractions 3 and fractions 4 provided enough material for a total of five iTRAQ experiments. The 4-plex experiments resulted in ~400 to ~1,500 protein identifications once the acceptance criterion of a ProteinPilot Unused Score of >1.3 (95% confidence) was implemented (Fig. 3). A subset of these was observed in more than a single dataset. The next stage of analysis involved the determination of the number of proteins with statistically significant changes as determined by the application of the random effects model of meta-analysis (Fig. 3). A total of 98, 45, 113, and 95 proteins were determined as having significant alcohol-induced alterations in protein content within the centrifugal fractions 1, 2, 3, and 4, respectively (see Supplemental Data for entire list of statistically significantly altered proteins, the centrifugal fraction, and numbers of iTRAQ experiments in which the protein identification were made, as well as the magnitude and direction of the change). Of these statistically significantly altered proteins, several were differentially regulated between the sexes as a result of long-term ethanol intake. In particular, a sexual dimorphism was revealed during analysis of canonical pathways of proteins involved in oxidative phosphorylation and mitochondrial dysfunction (Fig. 6). Proteins in complex I (NADH dehydrogenase), complex III (cytochrome b-c1), complex IV (cytochrome c oxidase), and complex V (ATPase) were also identified as being differentially expressed between males and females as a consequence of long-term ethanol intake.

**Proteomics: network analysis.** To place the proteomic findings into a biological context, pathway and network analysis was utilized (Ingenuity Systems, Redwood City, CA). In particular, a network of proteins involved in cardiovascular system development and function was created from proteins identified within fraction 4 (Fig. 4). Analysis of this network highlighted a sexual dimorphism in troponins, a regulatory protein required for contraction of the heart. The cardiac troponins were found to be upregulated in female rats consuming alcohol compared with female controls, whereas down-regulation was observed in alcohol treated male rats relative to their controls.

Western blot analysis was performed on troponin C and troponin I. The expression as indicated by iTRAQ analysis (Table 2) was consistent with Western results (Fig. 5). Statistical analysis demonstrated a significant alcohol-induced increase in troponin C expression in females and a concomitant decrease in troponin C expression in males (P < 0.001) (Fig. 5A). For troponin I there was a trend (P = 0.0584) of upregulation in females consuming alcohol, while males treated with alcohol showed no change compared with their respective vehicle control groups (Fig. 5B).

Numerous mitochondrial proteins were also identified as being differentially expressed between males and females as a consequence of long-term ethanol intake. In particular, a sexual dimorphism was revealed during analysis of canonical pathways of proteins involved in oxidative phosphorylation and mitochondrial dysfunction (Fig. 6). Proteins in complex I (NADH dehydrogenase), complex III (cytochrome b-c1), complex IV (cytochrome c oxidase), and complex V (ATPase) were also identified as being differentially expressed between males and females as a consequence of long-term ethanol intake.

### Table 1. Sex differences in the myocardial structure and function as measured by M-mode echocardiography in control and alcohol-fed rats

<table>
<thead>
<tr>
<th></th>
<th>Female Ctrl</th>
<th>Female EtOH</th>
<th>Male Ctrl</th>
<th>Male EtOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV mass, g</td>
<td>1.2 ± 0.1*</td>
<td>1.2 ± 0.1</td>
<td>1.5 ± 0.1</td>
<td>1.3 ± 0.1*</td>
</tr>
<tr>
<td>SV, ml/min</td>
<td>0.5 ± 0.03</td>
<td>0.4 ± 0.04</td>
<td>0.6 ± 0.03</td>
<td>0.4 ± 0.05*</td>
</tr>
<tr>
<td>CO, ml/min</td>
<td>229 ± 17</td>
<td>173 ± 23</td>
<td>305 ± 15</td>
<td>210 ± 25*</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>490 ± 8</td>
<td>489 ± 12</td>
<td>508 ± 9</td>
<td>476 ± 9</td>
</tr>
<tr>
<td>EF, %</td>
<td>99.8 ± 0.1</td>
<td>98.7 ± 0.6</td>
<td>98.6 ± 0.5</td>
<td>98.7 ± 0.4</td>
</tr>
<tr>
<td>FS</td>
<td>90.0 ± 1.2*</td>
<td>80.5 ± 2.8</td>
<td>81.2 ± 3.2</td>
<td>80.5 ± 2.5</td>
</tr>
<tr>
<td>EDD, cm</td>
<td>0.56 ± 0.01*</td>
<td>0.51 ± 0.02*A</td>
<td>0.65 ± 0.01</td>
<td>0.58 ± 0.02*</td>
</tr>
<tr>
<td>ESD, cm</td>
<td>0.05 ± 0.01*</td>
<td>0.10 ± 0.02</td>
<td>0.13 ± 0.02</td>
<td>0.11 ± 0.02</td>
</tr>
<tr>
<td>LVPW&lt;sub&gt;c&lt;/sub&gt;, cm</td>
<td>0.24 ± 0.01</td>
<td>0.26 ± 0.02</td>
<td>0.26 ± 0.01</td>
<td>0.24 ± 0.01</td>
</tr>
<tr>
<td>IVS&lt;sub&gt;c&lt;/sub&gt;, cm</td>
<td>0.16 ± 0.01</td>
<td>0.18 ± 0.01</td>
<td>0.18 ± 0.01</td>
<td>0.19 ± 0.01</td>
</tr>
</tbody>
</table>

Values shown are means ± SE for n = 5 in each group. LV mass, echocardiography-based left ventricular mass estimation; SV, stroke volume; CO, cardiac output; HR, heart rate; EF, ejection fraction; FS, fractional shortening; EDD, end-diastolic dimension; ESD, end-systolic dimension; LVPW<sub>c</sub>, left ventricular posterior wall thickness in diastole; IVS<sub>c</sub>, interventricular septum thickness in diastole. *P < 0.05 compared with respective control group; #P < 0.05 compared with control male; ΔP < 0.05 compared with ethanol male.

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1 The online version of this article contains supplemental material.
were identified and determined to be upregulated in males consuming alcohol compared with their control counterparts but either showed no statistically significant change or were downregulated in females consuming alcohol compared with their respective controls. The enzyme citrate synthase (CS) serves as the pace-making enzyme in the first step of the TCA cycle. CS showed a statistically significant increase (60%) in male alcohol rats compared with controls, although no change was observed between the female groups. Furthermore, two critical enzymes in fatty acid oxidation, carnitine palmitoyltransferase II (CPT2) and 2,4-dienoyl CoA reductase were upregulated in males consuming alcohol compared with their control counterparts. The enzyme citrate synthase (CS) catalyzes the second step in transmitochondrial membrane transport and activation of fatty acids for metabolism, was observed in males receiving the alcohol-containing diet. Both ethanol groups exhibited some echocardiographic evidence of the initiation of a compensatory response to the alcohol insult. However, more numerous and significant changes were found in the male alcohol group, suggesting that male myocardium is more susceptible to the toxic effects of alcohol at this early stage in the progression of AHMD to DCM. Although pressure and volume measurements were not taken, these findings are consistent with another study that demonstrated sex-dependent differences in heart function and structure as a consequence of long-term alcohol intake (31, 32). Piano et al. (32) showed that only the male ethanol group demonstrated evidence of a decline in cardiac performance after receiving alcohol for 8 mo, whereas neither females nor ovariectomized females showed a decline in function. In that study, sex-dependent differences in the degree of damage to the posterior wall thickness and IVS thickness were also reported, with greater thinning of these dimensions observed in males. Although the reduction in EDD in both sexes reported herein seems contrary to the development of alcohol-induced DCM, this may be indicative of an initial, early-stage compensatory response. Broadening future studies to include the potential role of hormones in the observed protein content alterations would allow a more complete picture of the alcohol-induced effects on the myocardium.

Our analysis revealed sexually dimorphic changes in myofibrillar proteins involved in cardiovascular development and required for contractile function. Specifically, an alcohol-induced upregulation of the troponins was observed in the alcohol-receiving females, while concomitant downregulation was observed in males receiving the alcohol-containing diet. The troponin complex, consisting of troponin I, troponin T, and troponin C, is located on the actin filament and is crucial not only for the calcium-mediated regulation of cardiac muscle

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**DISCUSSION**

In this paper we have shown that alcohol affects the male and female heart differently, in terms of observed structural and functional abnormalities and alterations in the expression of a broad array of proteins involved in cardiovascular function and development as well as energy-producing mitochondrial proteins. Specifically, the observed alterations in myocardial protein content suggest that female hearts are protected from the toxic effects of ethanol. In contrast, males appear to exhibit more serious structural abnormalities and altered expression of components of the contractile machinery and proteins that are linked to damaging superoxides. Women have a higher risk of alcohol-induced organ damage when higher blood alcohol levels are reported compared with men (38). However, in this model, blood alcohol levels are not statistically different between males and females, and this may help explain the observed protective effect in females. Although the effects described herein are observed in our model of chronic alcohol consumption, it is possible that the changes are not necessarily indicative of a causative effect of alcohol.

Both ethanol groups exhibited some echocardiographic evidence of the initiation of a compensatory response to the alcohol insult. However, more numerous and significant changes were found in the male alcohol group, suggesting that male myocardium is more susceptible to the toxic effects of alcohol at this early stage in the progression of AHMD to DCM. Although pressure and volume measurements were not taken, these findings are consistent with another study that demonstrated evidence of a decline in cardiac performance after receiving alcohol for 8 mo, whereas neither females nor ovariectomized females showed a decline in function. In that study, sex-dependent differences in the degree of damage to the posterior wall thickness and IVS thickness were also reported, with greater thinning of these dimensions observed in males. Although the reduction in EDD in both sexes reported herein seems contrary to the development of alcohol-induced DCM, this may be indicative of an initial, early-stage compensatory response. Broadening future studies to include the potential role of hormones in the observed protein content alterations would allow a more complete picture of the alcohol-induced effects on the myocardium.

Our analysis revealed sexually dimorphic changes in myofibrillar proteins involved in cardiovascular development and required for contractile function. Specifically, an alcohol-induced upregulation of the troponins was observed in the alcohol-receiving females, while concomitant downregulation was observed in males receiving the alcohol-containing diet. The troponin complex, consisting of troponin I, troponin T, and troponin C, is located on the actin filament and is crucial not only for the calcium-mediated regulation of cardiac muscle

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**Table 2. Selected proteins that demonstrate the dimorphism between male and female rats**

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Uniprot Identifier</th>
<th>FA:FC</th>
<th>MA:MC</th>
<th>FC:MC</th>
<th>FA:MA</th>
</tr>
</thead>
<tbody>
<tr>
<td>e&quot; transferring flavoprotein dh</td>
<td>Etfdh</td>
<td>Q66HF3</td>
<td>1.2</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>Isocitrate dehydrogenase 2</td>
<td>Idh2</td>
<td>P56574</td>
<td>0.7</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>Alpha B crystallin</td>
<td>Cryab</td>
<td>P23928</td>
<td>0.7</td>
<td>NC</td>
<td>1.8</td>
</tr>
<tr>
<td>Glutathione S transferase mu 2</td>
<td>Gstm2</td>
<td>P08010</td>
<td>NC</td>
<td>5.1</td>
<td>NC</td>
</tr>
<tr>
<td>Carnitine palmitoyltransferase II</td>
<td>Cpt2</td>
<td>P18886</td>
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<td>NC</td>
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<td>P70555</td>
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FC, female control; FA, female alcohol; MC, male control; MA, male alcohol; NC, no change.
contraction, but also for controlling the efficiency of power produced by the muscle (3, 19). Troponin, attached to tropomyosin, undergoes a calcium-induced conformational change that results in the movement of tropomyosin away from the active site of actin so that cross bridges can form between myosin and actin, thereby producing force and/or movement. Reduced troponin content would be associated with a reduced interaction of the myosin cross bridge and the actin filaments, which corresponds to decreased force production. The disparity seen in the level of the troponins, reported from the iTRAQ analysis and validated by Western blot techniques, may shed insight into understanding the echocardiography data that demonstrated a protection of the female rat myocardium to the negative functional and structural alterations associated with long-term alcohol intake. It may be that females are better able to adapt to the alcoholic insult in response to the upregulation of the troponins, whereas in males the expression of these were lower. Other potentially protective changes in female were observed as well.

Calmodulin, a regulator of CaM kinase II, is limiting within the heart and negative changes in its expression are associated with impaired cardiac growth (15), which is consistent with our findings. An elevation of calmodulin expression in the female heart following alcohol consumption is consistent with a protective effect in females, whereas the decreased expression observed in males may negatively impact calcium signaling. Recently, parathyroid hormone (PTH), which acts to increase calcium concentration by activating the parathyroid hormone receptor (PTHR), has been implicated in myocardial hypertrophy (27). Elevated PTH has been reported in patients with chronic heart failure (25) with an acceleration of a decompensation of the left ventricle, evidenced by reduced fractional shortening and posterior wall thickness, observed in PTH-treated mice (7). In this study, males consuming ethanol demonstrated an increase in PTH 2 receptor. It is tempting to speculate that changes in calmodulin and PTHR may affect calcium regulation and/or sensitivity and that this contributes to the sex-dependent differences in alcohol susceptibility in the heart.

Fig. 4. Protein network analysis. IPA-determined network of identified myofibrillar proteins involved in cardiovascular system development and function. The top network demonstrates the alterations in protein content between female alcohol and female control rats. The bottom network demonstrates the difference between male alcohol and male control rats. Specifically, the troponins are upregulated in female as a result of chronic alcohol consumption, while downregulated in male, compared with respective control groups (insets). Red, upregulated; green, downregulated.
Glutathione peroxidase 1 (Gpx1) reduces levels of cellular peroxide through its conversion to nonreactive products and thus is a key player in the cellular defense mechanism. In this study, Gpx1 was reduced in alcohol-receiving males, whereas females demonstrated no change in the expression of this protein. Consistent with the observations reported herein, an acceleration of dysfunction and elevated oxidative stress are observed with decreased Gpx1 activity (1). This increased susceptibility to dysfunction highlighted the significance of Gpx1 as an intrinsic antioxidative mechanism and the importance of its ability to maintain normal myocardial structure and function.

Fatty acids are the primary fuel source of the heart. Several steps are involved in fatty acid metabolism, including the activation and transport of fatty acids into the mitochondria, fatty acid beta-oxidation (FAO), and oxidative phosphorylation. Under normal conditions nearly all of the energy produced in the heart is a result of oxidative phosphorylation within the mitochondria (36). The bulk of fatty acid derivatives pass the mitochondrial inner membrane via a carnitine-mediated process where CPT2, together with carnitine palmitoyltransferase I, mediate translocation of the fatty acids into the mitochondria (39). A series of redox reactions, and the concomitant transfer of electrons, are carried out within five protein complexes. The rates of flux through this series of protein complexes are controlled, in part, by the degree of expression of key mitochondrial proteins (i.e., enzymes and transporters). As specific proteins mediate each of these stages of energy production (i.e., cardiac fatty acid uptake and transport, beta-oxidation, and oxidative phosphorylation), it is conceivable that either defects in the synthesis of these proteins or alterations in the content of these proteins may be related to cardiac dysfunction.

The data presented herein suggest that energy-producing metabolic processes in the male and female rat heart respond differently to chronic alcohol. This is evidenced, in part, by reported alcohol-induced increases in mitochondrial proteins involved in energy production in males but not females. Down-regulation of CPT2, as was observed in alcohol-receiving females, may indicate suppression in the transport of fatty acids into the mitochondria for subsequent FAO. In contrast, the upregulation of CPT2 in males consuming alcohol may allow increased delivery of FA into the mitochondrial lumen for oxidation. Increased FAO may result in greater transfer of electrons between molecules and the creation of superoxide containing molecules, which are well-recognized hallmarks of several conditions characterized by cardiac pathophysiology, such as heart failure (9). It is possible, then, that the females’ unchanged expression of FAO-related proteins with alcohol exposure may prevent the additional oxidative stress that is experienced in male rats, where expression of FAO-related proteins is increased, and thus be protective in the females.

The capacity for mitochondrial energy production is influenced by diet (e.g., alcohol consumption) (2, 23). As energy demands increase due, in part, to alterations in cardiac output, both mitochondria number and expression of mitochondrial proteins increase (23). Males may respond to the insult of the chronic alcohol-containing diet by increasing mitochondrial number, as suggested by the observed parallel increases in citrate synthase and proteins involved in the complexes of the oxidative phosphorylation pathway. This is consistent with previous observations made by Marin-Garcia et. al. (26), who reported an increase in mitochondrial DNA copy number in alcohol-treated rats compared with control rats.

As mitochondria can produce reactive oxygen species (ROS) through several mechanisms (28), one of which entails complex I leaking electrons to oxygen (9), an increase in mitochondrial number would be correlative to an increased capacity for generation of damaging ROS (5). Increased complex I protein content, as observed in males receiving the chronic alcohol diet, may be indicative of an increased flux and subsequent oxidative stress. The observed increases in mitochondrial enzyme activities reported herein are consistent with previously reported results of a study investigating the relationship between alcohol intake, myocardial enzyme activity, and myocardial dysfunction (33). In that study, activities of mitochondrial enzymes were all reported as higher in heavy drinkers and were correlated with alterations in functional parameters (e.g., cardiac output). Those results contrast with the results of an earlier study that reported an alcohol-induced depression of mitochondrial respiratory function in cardiac muscle (29). However, differences in study design, such as the species studied and duration of alcohol intake, may account for the contradictory findings. Although our interpretation of the observed sexually dimorphic response in selected proteins suggests that increased expression of oxidative-related proteins is correlated with diminished contractility, it is possible that these two mechanisms are independent of one another. A recent study...
investigating the relationship between mitochondrial dysfunction and oxidative damage supports our interpretation, suggesting that increased ROS production causes “preferential oxidation of myofibrillar proteins and provides a mechanistic link between oxidative damage and impaired contractility” through altered calcium sensitivity and efficiency of contraction (5). A time course of the specific alcohol-induced changes to myocardial protein content might prove useful in appreciating the inherent differences between males and females in the myocardium’s response to alcohol.

In this study, we also developed a random effects model of meta-analysis for combining multiple iTRAQ experiments to collectively analyze protein identifications. Either three or five iTRAQ experiments were combined for subsequent statistical analysis. An advantage to such an approach includes an increase in statistical power to detect an overall effect of chronic alcohol intoxication. A weighted average of the protein ratios, and the associated error factors for those identifications, obtained from each individual iTRAQ experiment provided a means to determine the overall effect of the alcohol insult on protein profiles in both male and female rats. The fractionation of the heart into centrifugal fractions, in conjunction with the ability to analyze each of the four study groups simultaneously, allowed the identification of low-abundance proteins and determination of the associated protein content differences between the sexes.

In summary, we have found a sex-dependent response in myocardial protein content as a consequence of long-term alcohol consumption. The observed alterations in mitochondrial proteins involved in oxidative phosphorylation and mitochondrial dysfunction reported in males may be indicative of a compensatory, or adaptive, response to alcohol. Males may adapt to the alcohol insult by trying to generate more energy to help protect the cell or to help the cell recover by upregulating key mitochondrial proteins involved in energy production but lack the ability to overcome the alcohol-induced insult to the myocardial tissue, leading to increased ROS production. A decrease in contractile efficiency as evidenced by reduced troponin content in conjunction with an ill-fated increase in the expression of key mitochondrial proteins may doom the male heart to the toxic effects of alcohol. In contrast, the female heart may be protected from the alcoholic insult due to its adaptive ability to maintain contractile abilities (i.e., increased troponin) while minimizing oxidative stress (i.e., decreased expression of ROS-generating proteins). This is consistent with the alterations in myocardial structure and function that are observed in males, but not females, following assessment via echocardiography. Future studies should consider the role of hormones in the protection/susceptibility of the heart to the development of alcohol-induced diseases.

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GRANTS

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DISCLOSURES

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

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