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Evidence of Altered Mitochondrial Protein Expression After Chronic Ethanol Consumption in the Aged Estrogen-Deficient Female Rat Heart

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Abstract

Background—Estrogen loss has been implicated to increase the risk of alcoholic cardiomyopathy in post-menopausal women. The purpose of this study was to identify novel mitochondrial protein targets for the treatment of alcoholic cardiomyopathy in aged women using a state-of-the-art proteomics approach. We hypothesized that chronic ethanol (EtOH) ingestion exacerbates maladaptive mitochondrial protein expression in the aged female heart.

Methods—Adult (3 mo) and aged (18 mo) F344 ovary-intact or ovariectomized (OVX) rats were randomly assigned an EtOH or control Lieber-DeCarli ‘all liquid’ diet for 20 wks. Proteomic analyses were conducted in mitochondria isolated from left ventricles using isobaric tags for relative and absolute quantification (iTRAQ) 8plex labeling and mass spectrometry (n=3–5/group).

Results—After EtOH, significant differences (false discovery rate < 5%) were observed in electron transport chain components (NADH dehydrogenase [ubiquinone] flavoprotein 2) as well as proteins involved in lipid metabolism (2,4 dienoyle-CoA reductase) and cellular defense (catalase), suggesting a possible link to congestive heart failure. Directional changes in protein levels were confirmed by Western blotting. Additionally, EtOH significantly reduced state 3 mitochondrial respiration in all groups, yet only reduced respiratory control index (RCI) in the aged OVX rat heart (p<0.05).

Conclusions—Collectively, the data reveal that EtOH-induced changes in the mitochondrial proteome exacerbate cardiac dysfunction in aged and estrogen-deficient hearts, but not in adult. In conclusion, iTRAQ is a powerful tool for investigating new mitochondrial targets of alcoholic cardiomyopathy.

Keywords

Lieber-DeCarli diet; left ventricle; ovariectomy; electron transport chain; iTRAQ

Introduction

Drinking alcohol in moderation is well-known to be beneficial to the heart resulting in reductions in cardiovascular death and total mortality (Di Castelnuovo et al., 2006, Kloner and Rezkalla, 2007, Lakshman et al., 2010). In addition, alcohol consumption mildly increases high density lipoprotein levels and decreases inflammatory markers such as C-reactive protein, fibrinogen, and interleukin-6 (Imhof et al., 2004, Maraldi et al., 2006, Suh et al., 1992). On the other hand, men and women who consume excessive alcohol over a prolonged period of time may be at risk for developing alcoholic cardiomyopathy (Fauchier et al., 2000, Laonigro et al., 2009). This disease can be divided into two stages, the asymptomatic phase leading to diastolic dysfunction and the symptomatic stage resulting in systolic dysfunction (Fernandez-Sola et al., 2000, Urbano-Marquez et al., 1989). Clinical studies have shown female alcoholics are more susceptible to acquiring cardiomyopathy than male alcoholics (Fernandez-Sola et al., 1997, Urbano-Márquez et al., 1995). While the mechanistic underpinnings of this heightened vulnerability to cardiomyopathy in females remains incompletely characterized, a possible role for estrogen receptors (ERs) has been suggested since left ventricular (LV) dysfunction induced by *acute* administration of ethanol (EtOH) is reversed by either ER α inhibition or ovariectomy (OVX) in female rats, through a downstream mechanism which limits ROS production and lipid aldehydes (Yao and Abdel-Rahman, 2016, El-Mas and Abdel-Rahman, 2014). Paradoxically, clinical features of chronic alcoholism are greater in aged women (Urbano-Márquez et al., 1995), and cardiac ER α levels are known to be reduced in estrogen deficient aged female rats (Novotny et al., 2009). There are a paucity of experimental studies in which females and *chronic* EtOH ingestion have been employed to address mechanisms underlying alcoholic cardiomyopathy, and fewer still which address advancing age into senescence (Fogle et al., 2011, Piano et al., 2005, Piano et al., 2007, Vary et al., 2007).

We have previously reported EtOH-induced reductions in protein synthesis of skeletal (Korzick et al., 2013) and cardiac (Lang and Korzick, 2014) muscle in aged females, an effect observed in adult male but not female rats following 26 weeks of EtOH consumption (Vary et al., 2007). Likewise, an eight month EtOH feeding study using adult male, female, and ovariectomized (OVX) female rats indicated a greater effect of EtOH in male and female OVX toward a phenotype of cardiomyopathy (Piano et al., 2007). This same study also noted the E₂ loss resulted in greater septal wall thinning and cardiac dysfunction (Piano et al., 2007). More recently, use of the proteomic approach isobaric tags for relative and absolute quantification (iTRAQ) and mass spectrometry revealed a phenotype whereby adult female rats are protected from alcoholic cardiomyopathy, while EtOH consumption damaged the adult male heart (Fogle et al., 2011). How aging in combination with E₂ loss affects the mitochondrial proteome in hearts of alcoholic females has not been investigated.

Cellular consequences of alcoholic cardiomyopathy include, but are not limited to, alterations in mitochondrial oxidative phosphorylation, calcium homeostasis, and oxidative stress (Laonigro et al., 2009, Ren and Wold, 2008). Myocardial cell death is associated with increased expression of apoptotic markers and activation of cell death signaling cascades (Fernandez-Sola et al., 2006). Given the essential role of mitochondria in regulating cell death pathways, myocardial ATP production, and cardioprotection (Murphy and

Steenbergen, 2007), we proposed to identify new mitochondrial protein targets for the possible treatment of alcoholic cardiomyopathy in aged women using a state-of-the-art proteomics approach. We hypothesized that chronic EtOH ingestion would exacerbate maladaptive mitochondrial protein expression in aged Fischer 344 (F344) rats (Lancaster et al., 2012), particularly with OVX. We conclude from proteomic screening that alcohol consumption revealed a loss of E₂-mediated protection in the female OVX heart, consistent with early signs of heart failure.

Materials and Methods

Animal Care

Adult (3 mo) and aged (18 mo) female F344 rats were housed individually under a 12 hr:12 hr light - dark cycle after being purchased from the NIA colony at Taconic (Hudson, NY). Rodents received standard rat chow (LabDiet 5001, PMI Nutrition International, St. Louis, MO) and water *ad libitum* upon arrival. A subset of adult and aged animals had bilateral OVX surgery and was allowed to recover for 4 weeks prior to the initiation of the EtOH feeding protocol. Rats were then subjected to the EtOH feeding protocol for 20 weeks (see below). At the time of sacrifice, uterine weights were recorded to confirm E₂ deficiency (Novotny et al., 2009, Hunter and Korzick, 2005), as the time to anestrus is variable in aged rats (Savonenko and Markowska, 2003, Sone et al., 2007). Animal protocols were reviewed and approved by the Pennsylvania State University Institutional Animal Care and Use Committee (IACUC) and followed accordingly.

Ethanol Feeding Protocol

Adult (n=38) and aged (n=42) female F344 rats, either intact or OVX, were fed a control Lieber-DeCarli 'all liquid' diet (Bio-Serv, Frenchtown, NJ) without EtOH for one week to adapt to the new feeding regimen (Sharda et al., 2012, Lieber et al., 1965, Lieber and Decarli, 1989). The Lieber-DeCarli diet consisted of 18% protein, 35% fat, and 47% carbohydrate, with the EtOH replacing carbohydrate (Lieber and Decarli, 1989). Rodents were subsequently randomized to control or alcohol groups for the duration of the 20 week study as described by us previously (Sharda et al., 2012, Lang and Korzick, 2014, Korzick et al., 2013). The alcohol group initially received 12% kcal from EtOH for one week, which was progressively increased until the maximum dose was given at week three (36% kcal derived from EtOH), remaining until the end of the study. The control group received an isocalorically-matched amount of dextrin in place of EtOH. Liquid diet consumption was recorded daily so animals could be pair-fed to account for EtOH-induced appetite suppression (Fujita et al., 2003). Control diet was administered isocalorically, using dextrin, based on consumption in each corresponding alcohol group. Characteristics of our model including blood alcohol levels and liver pathology are described elsewhere (Sharda et al., 2012).

Isolated Heart Preparation and Perfusion Protocol

The isolated heart preparation and perfusion protocol were previously described (Lancaster et al., 2011). Briefly, animals were anesthetized by sodium pentobarbital (40 mg/kg body wt, i.p.), and hearts were rapidly excised by midline thoracotomy and immediately rinsed in ice

cold (4 °C) saline. Within 60s of excision, hearts were secured via aortic cannulation to a Langendorff apparatus and perfused in a retrograde manner at constant pressure (85 mm Hg), temperature (37 °C), and pH (7.4) with a modified Krebs-Henseleit bicarbonate buffer (1.75mM CaCl₂, 117.4mM NaCl, 4.7mM KCl, 1.2mM MgSO₄, 1.3mM KH₂PO₄, 24.7mM NaHCO₃, 11.0mM glucose, 0.5mM pyruvate and 0.5mM EDTA, gassed continuously with 95% O₂–5% CO₂). A fluid-filled latex balloon was inserted into the left ventricle (LV) was inflated to yield an end diastolic pressure (EDP) of 5–6 mm Hg and LV were paced at 260 bpm. LV function was assessed using the Ponemah Physiology platform (Gould Instrument Systems, Valley View, OH). Hearts were perfused for 18 min to assess baseline function prior to iTRAQ sample preparation. Groups studied for function, iTRAQ, and biochemical experiments were adult, adult OVX, aged, and aged OVX +/- EtOH.

iTRAQ Sample Preparation

Mitochondrial fractions were obtained from the LV of adult and aged hearts by differential centrifugation exactly as described previously (Lancaster et al., 2011). Protein concentrations were determined according to the Bradford assay (Bradford, 1976). The iTRAQ 8Plex Reagent Kit (AB SCIEX, Foster City, CA) was used following the manufacturer's directions with minor exceptions (Lancaster et al., 2011). Briefly, 100 µg of a mitochondrial homogenate was concentrated and reconstituted in 0.5 M triethylammonium bicarbonate (TEAB) buffer. Samples were denatured with 2% SDS and reduced with 50 mM tris-(2-carboxyethyl)-phosphine (TCEP) before incubating for 1 hr at 60 °C. Cysteine blocking reagent (84mM iodoacetamide solution) was added to each sample and incubated for 30 min in the dark. Afterwards, trifluoroethanol was added to the cocktail and the homogenates were digested with sequencing grade trypsin (protein:trypsin ratio = 10:1) for 12–16 hr at 48 °C. Each iTRAQ reagent (113–119 and 121 m/z) was reconstituted in isopropanol, added to the corresponding sample (pH ~7.8). The labeling reaction incubated for 2 hr at room temperature, was quenched with ddH₂O, and then dried. The pellet was washed by resuspension in ddH₂O, and then dried completely. Two additional ddH₂O washes were performed before the pellet was resuspended in 12 mM ammonium formate in 25% acetonitrile, pH 2.5–3.0. Strong cation exchange (SCX) chromatography was performed followed by reversed-phase liquid chromatography (RP-LC) at the Penn State Hershey Proteomics Core Facility. Samples were spotted onto matrix-assisted laser desorption/ionization (MALDI) plates and analyzed in a data-dependent manner with a tandem time-of-flight instrument.

iTRAQ Data Analysis

Protein Pilot 4.0 software (ABI-MDS Sciex, Foster City, CA) using the Paragon algorithm was used to quantify protein levels based on the acquired MS/MS spectra (Lancaster et al., 2011, Shilov et al., 2007). The algorithm searched the NCBI nr (National Center for Biotechnology Information) protein database concatenated in reverse order, which is referred to as a decoy database. Proteins identified with a local false discovery rate of less than 5% using the PSPEP (Proteomics System Performance Evaluation Pipeline) beta software tool (ABI-MDS Sciex, Foster City, CA), were deemed acceptable protein identifications (Tang et al., 2008). If the protein expression value was greater than one, this corresponded to an increase in abundance, while values less than one represented a decrease

in expression. In addition, PANTHER (**P**rotein **A**nalysis **T**hrough **E**volutionary **R**elationships) analysis (Thomas et al., 2003) was performed to link protein identifications to biological processes as affected by chronic alcohol consumption.

Western Blotting

Equal amounts of mitochondrial homogenates were run on criterion SDS-PAGE gels (Bio-Rad, Hercules, CA) and transferred to PVDF membranes according to our standard laboratory procedure (Korzick et al., 2004). Membranes were blocked in 6% nonfat milk and incubated overnight with anti-catalase (sc-271803, Santa Cruz Biotechnology Inc., 1:5000 dilution). Afterwards, the blots were incubated with the corresponding HRP secondary antibody and developed using enhanced chemiluminescence reagent (GE Amersham, Piscataway, NJ). Scion Image software (NIH) was used for densitometry analysis, and SYPRO Ruby staining (Invitrogen, Grand Island, NY) of the membrane was performed to correct for protein loading (Ping et al., 1997).

Real-time PCR

RNA was isolated as previously described (Chomczynski and Sacchi, 1987, Lancaster et al., 2011). Samples were amplified (ABI model 7300) and analyzed using the 2^{-Ct} method with the reference gene cyclophilin (values were not different in alcohol treated groups). Forward and reverse primers for NADH dehydrogenase [ubiquinone] flavoprotein 2 (NDUFV2), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α), and atrial natriuretic factor (ANF) were as follows: NDUFV2, L-TACACCTTGCATGCTTCGAG, R-TCCTTGCGGTGTCAGATCCTC; PGC-1 α , L-ATGTGTCGC CTTCTTGCTCT, R-ATCTACTGCCTGGGGACCTT; ANF, L-GGGGGTAGGATTGACAGGA T, r-CTCCAGGAGGGTATTACCA.

Mitochondrial Isolation for Respiration

Hearts were excised and secured to a Langendorff apparatus as described above. Following a 3 min perfusion with a modified Krebs-Henseleit buffer, hearts were removed from the apparatus, ventricles were isolated and homogenized in 3ml of STE buffer (300mM sucrose, 10mM Tris (pH 7.4), 2mM EGTA (pH 7.4)). Homogenates were diluted in STE buffer supplemented with 0.5% BSA and centrifuged at 2000 $\times g$. The resultant supernatant was centrifuged at 10,000 $\times g$ and the mitochondrial pellet was washed twice in STE buffer prior to resuspension in 300 μ l STE buffer. Protein concentration was determined by the method of Bradford (Bradford, 1976). Groups studied for mitochondrial experiments were adult, aged, and aged OVX +/-EtOH.

Mitochondrial Respiration

Respiration of isolated mitochondria was measured at 30°C using a Clarke-type electrode attached to a YSI oxygraph (Yellow Springs, Ohio) as described previously (Hunter et al., 2012). Briefly, mitochondria were incubated in a buffer containing (in mM): 125 KCl, 20 MOPS, 10 Tris, 2 MgCl₂, 2 KH₂PO₄, 0.5 EGTA, pH 7.2. Complex I respiration was measured in the presence of 2.5mM α -ketoglutarate + 1 mM malate; state 3 respiration was

initiated by addition of 1 mM ADP. Respiratory Control Index (RCI) was determined by a ratio of state 3: state 2 respiration.

Statistical Analyses

For the proteomic analysis, data are presented as protein expression ratios, p values, and error factors as described previously (Lancaster et al., 2011). Quantitative protein ratios were expressed relative to a pooled adult control sample (three adult ovary-intact control diet hearts that were independently run and analyzed) which were included in each of the five iTRAQ 8plex analyses. The ProteinPilot 4.0 software program (ABI-MDS Sciex, Foster City, CA) using the Paragon algorithm was used to perform the statistical analysis on the iTRAQ data. Specifically, $p < 0.05$ was considered statistically significant between groups. For morphological, functional, Western blotting, RTqPCR, and mitochondrial function results, the data are presented as means $\pm$ SE and analyzed using the Statistical Analysis System (SAS) general linear model with three-way ANOVA (age $\times$ ovary status $\times$ diet). To assess interaction effects with respect to age, ovary status, and diet, higher order terms were utilized. Post hoc analysis was performed using the Tukey-Kramer method and $p < 0.05$ was considered statistically significant between groups.

Results

Baseline Morphology and LV Function

As expected, end of study rat weight was reduced for EtOH-fed vs. control-fed animals, reaching significance in aged (age*diet interaction; Table 1). Because animals were pair-fed throughout the duration of the study, total weight gain throughout the 20 week study was less in EtOH fed-rats, with greatest effects observed in aged (age*diet interaction; Table 1). While careful effort was given to match BW to total daily dietary consumption across groups, a small but significant decrease in EtOH consumption was also observed in aged vs adult when normalized to BW (Table 1). Importantly, BAC is similarly increased in adult and aged (Sharda et al., 2012). LV weight was increased in aged animals only (Table 1), a pattern which persisted when normalized to tibial length (data not shown). Baseline LV developed pressure (LVDP) was significantly reduced in aged animals and an ovary status by diet interaction revealed that LVDP was reduced in alcohol consuming OVX rats. Baseline LV developed pressure (LVDP) was reduced in aged animals and an ovary status by diet interaction revealed that LVDP was reduced in alcohol consuming OVX rats. Significant reductions were observed in LV - dP/dt in ethanol treated aged OVX, with a tendency for reduction in LV +dP/dt in this same group (Table 1). Reductions in coronary flow rate normalized to LV weight were observed in aged and EtOH treated animals, respectively (Table 1: main effects of age and EtOH treatment).

iTRAQ Analysis of Mitochondrial Proteins from LV Following Alcohol Ingestion

To assess mitochondrial protein levels after chronic EtOH consumption in LV isolated from adult and aged, n=5 8plex iTRAQ analyses were conducted. A total of 493 unique proteins were identified between the individual runs. Proteins of interest were chosen, based on whether levels were significantly affected by alcohol in one of the four experimental groups: adult ovary-intact, adult OVX, aged ovary-intact, and aged OVX. A total of 31

mitochondrial proteins were modified by chronic EtOH consumption across groups, of which n=26 are novel observations and not reported previously (indicated by an * in Tables 2, 3, and 4). Specifically, 16 proteins (~50%) had altered levels after alcohol consumption in the adult intact and/or OVX groups only (n=8 for adult, n=5 for adult OVX, n=3 both adult and adult OVX; Table 2). Primarily, this subset of proteins was downregulated with alcohol intake and notable examples include: stress-70 protein, aconitate hydratase, D-beta-hydroxybutyrate dehydrogenase, amine oxidase A, glutathione peroxidase 1, and enoyl-CoA hydratase. On the other hand, Table 3 depicts the four proteins specifically modified by EtOH ingestion in the aged intact and/or OVX. These proteins comprised mainly electron transport components such as electron transfer flavoprotein subunit alpha and beta, NADH dehydrogenase [ubiquinone] flavoprotein 2 (NDUFV2), and acetyl-CoA acetyltransferase. Notably, NDUFV2 levels were decreased by ~50% with alcohol in both the aged intact and aged OVX groups. Finally, Table 4 summarizes the 11 (~33%) proteins affected by alcohol in all experimental groups. With the exception of calsequestrin-2 precursor, the magnitude and direction of change in protein levels affected by chronic EtOH administration was similar in adult and aged. Catalase, a well-known antioxidant was chosen for Western blotting to confirm the iTRAQ results. As shown in Figure 1, catalase was upregulated with alcohol consumption in all experimental groups.

Candidate proteins of interest were then classified by biological function using PANTHER ontological analysis (Figure 2A and 2B). Of the proteins affected by alcohol consumption in the adult intact and/or adult OVX groups together, oxidative phosphorylation components and proteins involved in lipid metabolism were the two largest clusters identified from the PANTHER analysis (Figure 2A and 2B). Mitochondrial subunits housed at the inner membrane and associated with ATP production were observed to have either increased or decreased levels depending on the subunit. Likewise, a similar trend was noted for the four proteins playing a role in lipid metabolism – 2,4 dienoyl-CoA reductase, acyl carrier protein, D-beta-hydroxybutyrate dehydrogenase, and medium-chain specific acyl-CoA dehydrogenase (Tables 2 and 4). Proteins directly altered in the aged intact and/or aged OVX groups with chronic EtOH ingestion (Figure 2B) were dominated by components of the electron transport chain and the expression pattern appeared to be bi-directional (Tables 3 and 4). The remainder of the mitochondrial proteins in the adult and aged groups fell into much smaller categories of interest including: tricarboxylic acid cycle, protein metabolism and modification, apoptosis, carbohydrate metabolism, and cellular defense (Figure 2A–B). Several abundant muscle proteins (myosin light chain 3, alpha-crystallin B chain, and creatine kinase M-type) were also detected in the iTRAQ screens as well as calcium-related proteins (calsequestrin-2 precursor and sarcoplasmic/endoplasmic reticulum calcium ATPase 2 isoform b).

Evaluation of mRNA levels from LV Following Alcohol Ingestion

To determine if the proteomic changes revealed by iTRAQ were downstream of genomic differences, RTqPCR was performed. NDUFV2 mRNA was unchanged with EtOH consumption in aged and aged OVX despite reduced protein levels (Figure 3A). PGC-1 α increased with EtOH consumption in all groups, regardless of ovary status, increasing by 70%, 32%, 24%, and 17% in adult, adult OVX, aged, and aged OVX, respectively (Figure

3B). ANF mRNA was significantly increased with age in all groups regardless of diet. Interestingly, the magnitude of change was greatly reduced with OVX in control-fed animals (325% vs 76%), while EtOH led to similar increases in ovary intact and OVX (186% vs 136%) (Figure 3C).

Mitochondrial Respiration

Complex I state 3 respiration rate was significantly decreased by EtOH consumption in adult, aged, and aged OVX mitochondria (Figure 4A). Notably, the slight, but non-significant reductions in state 2 respiration in adult and aged intact groups (Figure 4B) preserved RCI, while aged OVX suffered decreased RCI aged OVX (Figure 4C) with EtOH consumption.

Discussion

Following administration of the EtOH-containing Lieber-DeCarli 'all liquid diet' for 20 weeks to adult and aged female F344 rats with and without E₂ deficiency, major summary findings include the identification of n=23 cardiac proteins not previously associated with chronic EtOH consumption in adult and/or aged female rats. Age-related decreases in cardiac function were observed, and EtOH consumption led to additional functional declines in estrogen deficient animals compared to their control-fed counterparts. Alterations in several key mitochondrial proteins related to chronic heart failure were noted in aged. Specifically, we observed significant reductions in NDUFV2 and acetyl-CoA acetyltransferase protein levels (Table 3) in conjunction with reduced LVDP and mitochondrial RCI in aged OVX following chronic EtOH consumption. Moreover, protein changes associated with E₂ deficient groups were also apparent, suggesting important interactions with chronic EtOH use. Collectively, these data provide novel evidence for exacerbated cardiac dysfunction with chronic EtOH consumption in estrogen-deficient adult and aged females with evidence to suggest a mitochondria-centric mechanism for a failing phenotype.

Previous proteomic findings have suggested cardioprotection with long-term alcohol ingestion in adult female F344 rats (Fogle et al., 2011, Vary et al., 2007) frequently explained by the French paradox. That E₂ reportedly protects from alcoholic cardiomyopathy (Piano et al., 2007) is supported herein by the lack of a detrimental EtOH effect on cardiac function in adult females. With OVX, however, we observed exacerbated injury and protein changes in mitochondrial electron transport chain (ETC) complexes, providing a potential link to E₂-deficiency. Protein subunits of the ETC complex I (NADH dehydrogenase), III (cytochrome bc₁ complex), and IV (cytochrome c oxidase) were altered by chronic EtOH consumption as detected by the iTRAQ analyses. For example, cytochrome c oxidase subunit 5A levels decreased with EtOH consumption in adult OVX vs adult ovary-intact rats similar to prior reports from adult males (Fogle et al., 2011). Notably, NADH dehydrogenase 1 alpha subcomplex subunit 7 responded inversely to EtOH in adult intact and OVX possibly identifying an interaction between E₂ and EtOH leading to proteomic responses in mitochondria that may underscore reduced LV function in adult OVX rats ingesting EtOH. In addition, downregulation of enzymes involved in carbohydrate

metabolism and the tricarboxylic acid cycle in conjunction with alterations in proteins subserving lipid metabolism suggest compensatory shifts in substrate usage given the metabolic burden of a high fat diet. The observed protein alterations by EtOH ultimately resulted in preserved mitochondrial RCI despite decreased state 3 respiration in adult suggesting no loss in mitochondrial efficiency of ATP production highlighting maintained mitochondrial function.

Proteins related to oxidative stress were also affected by chronic EtOH in the adult heart. In particular, EtOH induced reductions in heat shock protein (hsp) 10 and 60 in adult OVX and hsp70 in adult intact. The varying degrees of cardiac dysfunction between groups may provide different implications for the observed hsp responses. Reduced hsp10 and 60 in adult OVX in the face of reduced LVDP may imply a dysfunctional mitochondrial unfolded protein response (UPR_{mt}), which has been shown to activate upon alcoholic stress (Ji, 2015). The UPR_{mt} upregulates chaperones to unfold or prevent aggregation of misfolded proteins, and antioxidants to reduce reactive oxygen species (ROS) in dysfunctional mitochondria (Pellegrino et al., 2013). OVX may disrupt necessary signaling via ER α (Ji, 2015, Pellegrino et al., 2013). Interestingly, similar reductions in hsp 60 and 70 were observed in adult male rats after EtOH consumption further supporting a role for E₂ signaling (Fogle et al., 2010, Patel et al., 1997). Alternatively, decreased hsp70 and glutathione peroxidase 1 (GPX-1, an antioxidant) in adult intact animals in the absence of reduced cardiac function may indicate sufficient mitochondrial protection and no need for UPR_{mt} activation. Decreased monoamine oxidase A in adult, an outer mitochondrial membrane source of ROS known to be increased with age (Lancaster et al., 2012), may also contribute to a cardioprotective phenotype in adult ovary-intact females following EtOH consumption. Increased PGC-1 α mRNA observed herein with EtOH consumption may be indicative of efforts to increase mitochondrial biogenesis to compensate for stress-induced mitochondrial dysfunction (Fernandez-Marcos and Auwerx, 2011). Interestingly, the magnitude of increase was sequentially blunted from adult intact to aged OVX suggesting a reduced ability to respond. Finally, the antioxidant, catalase, was increased by EtOH in support of UPR_{mt} suppression (Wu et al., 2010). This finding is not surprising since previous evidence of resveratrol-mediated catalase activity upregulation has been implicated in cardioprotective responses (Das et al., 2011).

Several early markers of heart failure (Hollander et al., 2011) following chronic EtOH ingestion were observed in aged females. ANF mRNA is increased with age suggesting a shift toward a failing phenotype (Sergeeva and Christoffels, 2013). After chronic EtOH consumption, calsequestrin-2 precursor levels are reduced which may limit calcium storage capacity (Hu et al., 2011) further contributing to EtOH-induced failure in the aged. Aged hearts similarly displayed variation in mitochondrial subunit protein levels in response to EtOH consumption, including changes in subunits of complex I (NADH dehydrogenase), III (cytochrome bc₁ complex), and IV (cytochrome *c* oxidase) as well as electron transfer flavoproteins previously associated with EtOH consumption (Fogle et al., 2010, Fogle et al., 2011).

Of clinical relevance to the field of alcoholic cardiomyopathy, were observations related to changes in the flavoproteins of Complex I. NADH dehydrogenase [ubiquinone] flavoprotein

2 (NDUFV2) was reduced by 50% in both aged intact and aged OVX groups after EtOH consumption vs adult. Interestingly, a proteomic screen of the aging male F344 left ventricle detected reduced levels of this same flavoprotein (Grant et al., 2009). A mutation in flavoprotein 2 is linked with early onset hypertrophic cardiomyopathy and encephalopathy, as the majority of the protein is unable to translocate to the mitochondria due to a disrupted mitochondrial localization signal (Bénit et al., 2003), which is supported by no change in mRNA levels herein. Furthermore, it has been suggested NDUFV2 acts as a scaffolding protein to ensure NDUFV1 is assembled correctly and is needed for a functional Complex I (Almeida et al., 1999) likely responsible for the observed reductions in state 3 respiration. Notably, NDUFV2 was reduced with age, while decreased mitochondrial RCI was observed in aged OVX. As such, alcohol ingestion may impair ATP production and support ROS generation by targeting Complex I in the aged E₂-deficient female population promoting reduced LVDP and early symptoms of heart failure. Decreased mitochondrial respiration has also been observed in adult male rats following chronic EtOH consumption (Weishaar et al., 1977, Segel et al., 1975), but whether detrimental mitochondrial function is a cause or consequence of the observed decreases in cardiac function remains to be determined.

Yet, in spite of these complications, two other classes of proteins were positively regulated possibly to compensate for mitochondrial dysfunction. Enzymes responsible for metabolizing lipids were again increased with EtOH consumption. For example, 2,4 dienoyl-CoA reductase, which is involved in β -oxidation of unsaturated fatty acids (Hiltunen and Qin, 2000). Upregulated 2,4 dienoyl-CoA reductase was also noted in adult male Sprague-Dawley rats after a long-term alcohol consumption experiment, but was of unknown origin or function (Fogle et al., 2010). Interestingly, this response to EtOH may be facilitated by the high fat diet, as all rats received the same calories from fat. Therefore, the ability for EtOH to increase lipid peroxidation may induce an upregulation of enzymes necessary to increase fatty acid beta oxidation and mitochondrial metabolism as seen in the pancreas (Sato et al., 2013). In addition, the cellular defense enzyme, catalase, was elevated with alcohol treatment in all ages likely as a compensatory mechanism for cardioprotection (Dai et al., 2010, Zhang et al., 2003). It is possible that increased catalase activity in response to ethanol may counter lipid peroxidation, as previously observed (Ojeda et al., 2012). Interestingly, these findings contrast prior findings of reduced catalase levels in the aging female F344 myocardium fed a normal diet (Lancaster et al., 2012), highlighting an EtOH-specific response.

In conclusion, quantitative proteomics by state-of-the-art iTRAQ 8plex labeling is a powerful tool for exploring differences in protein levels contributing to alcoholic cardiomyopathy. The results herein are the first to demonstrate a phenotypic shift in the cardiac mitochondrial proteome consistent with heart failure in aged females following chronic EtOH consumption. EtOH-induced alterations in ETC subunits with advancing age may underlie, at least in part, decreased mitochondrial efficiency and in turn, worsened cardiac function. Our findings further identify candidate proteins associated with oxidative phosphorylation (NDUFV2), oxidative stress (hsp 10 and 60), and calcium handling (calsequestrin-2) as targets for future intervention in the treatment of alcoholic cardiomyopathy in post-menopausal women.

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Figure 1.

Catalase levels are increased with chronic EtOH consumption. Representative immunoblots are presented for mitochondrial catalase levels from each of the eight groups (n=3–5/group).

†Effect of alcohol vs control diet, *effect of OVX within age group, $P < 0.05$. Values are means $\pm$ SE; data are expressed relative to control adult intact. Alc, alcohol group; Ctrl, control group; and Ovx, ovariectomy.

A. Proteins Affected by Alcohol in Adult Groups

B. Proteins Affected by Alcohol in Aged Groups

Figure 2. Panther representation of the unique proteins identified in iTRAQ 8plex analyses in adult (A) and aged (B) groups with chronic EtOH consumption, grouped according to biological function.

Figure 3.

Select changes in mRNA induced by age or EtOH consumption. Left ventricular levels of NADH dehydrogenase [ubiquinone] flavoprotein 2 (NDUFV2), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α), and atrial natriuretic factor (ANF) relative to housekeeping gene cyclophilin D (CyD) (n=3–5/group). [†]Effect of alcohol vs control diet, [‡] Main effect of age, $P < 0.01$. Values are means $\pm$ SEM; OVX, ovariectomy.

Figure 4.

Mitochondrial state 3 respiration is reduced with chronic EtOH consumption. Complex I respiration rates in mitochondria isolated from rat heart. [†]Effect of alcohol vs control diet, $P < 0.05$. Values are means $\pm$ SEM; RCI, respiratory control index; OVX, ovariectomy.

Table 1

Baseline morphology and functional characteristics after administering the Lieber-DeCarli diet.

	Control				Alcohol				Main Effects
	Adult	Adult OVX	Aged	Aged OVX	Adult	Adult OVX	Aged	Aged OVX	
n	13	5	10	7	14	6	12	13	
	256.9 ± 3.1	298.0 ± 6.2	415.8 ± 6.2	418.1 ± 13.6	230.8 ± 4.0	295.0 ± 4.6	353.0 ± 9.8	372.4 ± 6.5	<i>d,e</i>
	63.0 ± 3.1	83.8 ± 3.3	121.8 ± 1.7	135.9 ± 4.6	40.1 ± 2.0	75.7 ± 4.1	68.6 ± 5.6	89.6 ± 4.4	<i>b,d</i>
Daily Consumption % BW (%)	18.1 ± 0.2	15.4 ± 0.3	15.5 ± 0.3	15.3 ± 0.4	19.0 ± 0.3	17.5 ± 0.4	15.9 ± 0.2	16.0 ± 0.2	<i>d,e</i>
Daily EtOH Consumption % BW (%)	nd	nd	nd	nd	0.97 ± 0.01	0.90 ± 0.02	0.82 ± 0.01	0.82 ± 0.01	<i>e</i>
	0.68 ± 0.06	0.08 ± 0.01	0.57 ± 0.03	0.19 ± 0.01	0.64 ± 0.03	0.07 ± 0.01	0.68 ± 0.03	0.19 ± 0.01	<i>b</i>
n	4	5	4	4	7	6	5	5	
LVW (g)	0.62 ± 0.02	0.62 ± 0.01	0.93 ± 0.02	0.79 ± 0.05	0.60 ± 0.01	0.60 ± 0.01	0.77 ± 0.02	0.78 ± 0.04	<i>d</i>
Flow/LVW (ml/min/g)	16.6 ± 1.13	15.6 ± 0.42	14.8 ± 0.73	16.6 ± 1.07	16.3 ± 1.44	14.8 ± 0.48	14.2 ± 0.66	13.5 ± 0.61	<i>a,c</i>
EDP (mmHg)	5.37 ± 0.5	5.82 ± 0.2	5.63 ± 0.2	6.39 ± 0.5	5.37 ± 0.1	5.61 ± 0.2	5.82 ± 0.4	5.63 ± 0.1	
	131.90 ± 3.5	141.22 ± 3.0	124.69 ± 1.3	125.89 ± 3.1	129.22 ± 2.4	122.94 ± 2.9	126.04 ± 2.4	122.53 ± 2.8	<i>d,f</i>
+dP/dt (mmHg/s)	3418 ± 3.7	3479 ± 116	3264 ± 128	3305 ± 240	3354 ± 145	3016 ± 85	3216 ± 123	2984 ± 117	
-dP/dt (mmHg/s)	2087 ± 157	2087 ± 89	2131 ± 158	2231 ± 192	2078 ± 117	1783 ± 49	1982 ± 139	1836 ± 124	<i>d</i>

Ovx, OVX; LVW, left ventricle weight; BW, body weight; BAC, blood alcohol content; EDP, end diastolic pressure; LVDP, left ventricular developed pressure; dP/dt, first derivative of LVDP curve.

* p<0.05.

^a age main effect;^b OVX main effect;^c EtOH main effect;^d age*EtOH interaction;^e age*OVX interaction;^f OVX*EtOH interaction.

Table 2

Proteins affected by chronic EtOH consumption in adult groups only. Alc, alcohol group; Ctrl, control group; and ovariectomy, OVX.

Accession #	Protein	iTRAQ Ratio for Alc Adult Groups vs. Ctrl Adult Intact
gi 158635 97 5	sarcoplasmic/endoplasmic reticulum calcium ATPase 2 isoform b	1.14 (Ovx)
gi 325974480	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 7	1.38 (Intact) , 0.87 (Ovx)
gi 40786469	dihydrolipoyl dehydrogenase, mitochondrial precursor	0.91 (Ovx)
gi 154816168	stress-70 protein, mitochondrial	0.78 (Intact)
gi 6981052	10 kDa heat shock protein, mitochondrial	0.75 (Ovx)
gi 206597443	60 kDa heat shock protein, mitochondrial	0.80 (Ovx)
gi 40538860	aconitate hydratase, mitochondrial precursor	0.77 (Intact)
gi 62079055	isocitrate dehydrogenase [NADP], mitochondrial precursor	0.76 (Intact), 0.86 (Ovx)
gi 158749584	succinyl-CoA ligase [ADP-forming] subunit beta, mitochondrial	0.44 (Intact)
gi 6978661	creatine kinase M-type	0.86 (Intact)
gi 55742813	D-beta-hydroxybutyrate dehydrogenase, mitochondrial	0.79 (Intact), 0.79 (Ovx)
gi 292494885	medium-chain specific acyl-CoA dehydrogenase, mitochondrial precursor	0.88 (Intact)
gi 270288740	amine oxidase [flavin-containing] A	0.88 (Intact)
gi 145275165	glutathione peroxidase 1	0.83 (Intact)
gi 17530977	enoyl-CoA hydratase, mitochondrial precursor	0.78 (Ovx)
gi 78365255	dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial	0.88 (Intact)

Table 3

Proteins affected by chronic EtOH consumption in aged groups only. Alc, alcohol group; Ctrl, control group; and ovariectomy, OVX.

Accession #	Protein	iTRAQ Ratio for Alc Aged Groups vs. Ctrl Adult Intact
gi 57527204	electron transfer flavoprotein subunit alpha, mitochondrial precursor	1.10 (Intact)
gi 51948412	electron transfer flavoprotein subunit beta	1.49 (Ovx)
gi 51092268	NADH dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial precursor	0.58 (Intact) , 0.55 (Ovx)
gi 8392836	acetyl-CoA acetyltransferase, mitochondrial precursor	0.87 (Ovx)

Table 4

Proteins affected by chronic EtOH consumption in adult and aged groups together. Alc, alcohol group; Ctrl, control group; and ovariectomy, OVX.

Accession #	Protein	iTRAQ Ratio for Alc Adult Groups vs. Ctrl Adult Intact	iTRAQ Ratio for Alc Aged Groups vs. Ctrl Adult Intact
gi 6981240	myosin light chain 3	1.27 (Ovx)	1.20 (Ovx)
gi 16905067	alpha-crystallin B chain	1.18 (Ovx)	1.20 (Ovx)
gi 6978607	catalase	1.20 (Intact)	1.33 (Intact)
gi 292781228	2,4-dienoyl-CoA reductase, mitochondrial precursor	1.25 (Ovx)	1.20 (Ovx)
gi 157820787	acyl carrier protein, mitochondrial	1.13 (Intact)	1.22 (Intact)
gi 55741544	cytochrome b-c1 complex subunit 2, mitochondrial	1.16 (Ovx)	1.30 (Ovx)
gi 68341999	cytochrome b-c1 complex subunit 8	1.33 (Intact)	1.30 (Ovx)
gi 198278533	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 9, mitochondrial	0.48 (Intact)	0.78 (Intact), 0.30 (Ovx)
gi 24233541	cytochrome c oxidase subunit 5A, mitochondrial precursor	0.86 (Ovx)	0.80 (Ovx)
gi 145651820	methylmalonate-semialdehyde dehydrogenase [acylating] mitochondrial precursor	0.80 (Intact)	0.88 (Intact), 1.20 (Ovx)
gi 157951709	calsequestrin-2 precursor	1.21 (Ovx)	0.92 (Intact)