CALL FOR PAPERS | *Cardiovascular Mitochondria and Redox Control in Health and Disease*

Chronic plus binge ethanol feeding induces myocardial oxidative stress, mitochondrial and cardiovascular dysfunction, and steatosis

Csaba Matyas,1,2 Zoltan V. Varga,1 Partha Mukhopadhyay,1 Janos Paloczi,1 Tamas Lajtos,1 Katalin Erdelyi,1 Balazs T. Nemeth,1 Mintong Nan,1 Gyorgy Hasko,3 Bin Gao,4 and Pal Pacher1
1Laboratory of Cardiovascular Physiology and Tissue Injury, National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Bethesda, Maryland; 2Heart and Vascular Center, Semmelweis University, Budapest, Hungary; 3Department of Surgery, Rutgers New Jersey Medical School, University Heights, Newark, New Jersey; and 4Laboratory of Liver Diseases, National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Bethesda, Maryland

Submitted 7 March 2016; accepted in final form 15 April 2016

**NEW & NOTEWORTHY**

Herein we describe new mouse models of cardiomyopathies induced by chronic plus single or multiple ethanol binges [National Institute on Alcohol Abuse and Alcoholism (NIAAA) models], which are characterized by impaired cardiovascular performance, mitochondrial dysfunction, and cardiac steatosis. Accordingly, the chronic plus binge ethanol feeding NIAAA mouse models are good candidates for the future investigations targeting alcoholic cardiomyopathy.

Alcohol has been consumed in many cultures for centuries, being a most often used psychoactive substance. According to recently published data, chronic alcoholism along with its harmful effects ranks among the top five risk factors for disease and death (26). Whether regular alcohol consumption has beneficial or harmful effects is still a subject of intensive debates. Although low to moderate daily consumption is considered to have favorable consequences on cardiovascular mortality (5, 19), chronic and heavy drinking might lead to cardiac dysfunction or subsequent heart failure (30) and increases the risk of sudden cardiac death (15). Long-term, heavy alcohol consumption leads to the development of alcoholic cardiomyopathy, a specific heart disease associated with characteristic tissue injury and histological and functional alterations (34).

Several contributing factors have been identified in the development of alcoholic cardiomyopathy such as oxidative and nitrative stress, myocardial hypertrophy, acetaldehyde protein adduct formation, apoptosis, increased activation of angiotensin II - angiotensin II receptor signaling, and fibrotic remodeling (34).

During the metabolism of ethanol (EtOH) it is degraded by the alcohol-dehydrogenase (ADH) or cytochrome P-450 2E1 (CYP2E1) enzymes into acetaldehyde (55). Because cardiac tissues have very low levels of ADH and CYP2E1 (1), EtOH may exert direct toxic effects in cardiomyocytes by interfering with the cardiac contractile protein synthesis or by inhibition of the enzymes of the citrate cycle and disturbance of the main
mitochondrial biogenesis regulator, the peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α) (46). The main product of EtOH metabolism is acetaldehyde, whereas reactive oxygen species (ROS) are formed as by-products. Acetaldehyde is a highly reactive molecule that binds to proteins or other macromolecules forming protein-adducts, which may further aggravate the toxic effects of EtOH (4). Superoxide anion bind to nitric oxide (NO) forming the highly reactive molecule peroxynitrite (32). Peroxynitrite then binds to different structural and contractile proteins and enzymes that contributes to subsequent cellular dysfunction (32). In chronic alcoholism, excessive NO production was found with increased endothelial and inducible NO synthase levels (8), which might further contribute to the oxidative/nitrosative stress.

It is noteworthy, that binge drinking is the most common form of alcohol abuse in cases of young adults, according to recently published data in 2013 (38). Binge drinking was reported to exert adverse cardiovascular effects including macro- and microvascular dysfunction (11), increased atherosclerotic plaque development (27), coronary calcification (35), and myocardial injury (52, 54). Although, there are several studies in the literature that investigate the effects of either long-term (16, 17, 21, 43) or acute (12, 18, 25, 40) EtOH consumption, animal models that are mimicking the human drinking patterns are limited. Bertola et al. (3) reported a mouse model of chronic plus binge EtOH feeding, the so-called NIAAA (National Institute on Alcohol Abuse and Alcoholism) model, which was developed to conform with human drinking behavior often seen in chronic alcoholics.

In this study we aimed to develop mouse models of alcoholic cardiomyopathies induced by chronic and binge ethanol (EtOH) feeding based on NIAAA mouse alcohol model, and characterize in detail cardiovascular function and pathological and biochemical alterations in these models.

METHODS

Animals. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Experiments were reviewed and approved by the Institutional Animal Care and Use Committee of the NIAAA. Forty-two young male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) at the age of 12–14 wk were included in the study and were kept in a specific pathogen-free animal facility under constant temperature (22 ± 2°C), humidity and with 12 h alternating light cycles.

Experimental protocol and treatment groups. Mice were fed according to the modified NIAAA model described previously (3). In brief, all mice were fed the Lieber-DeCarli liquid diet (Bio-Serv, Frenchtown, NJ) ad libitum for the first 5 days as acclimatization. Then, the feeding protocol was switched to one of the following seven protocols (Fig. 1A): for chronic feeding: 1) free access to ethanol (EtOH) Lieber-DeCarli (Bio-Serv) diet containing 5% (vol/vol) EtOH for 10 days (EtOH 10d, n = 6); 2) for 20 days (EtOH 20d, n = 5); 3) for 40 days (EtOH 40d, n = 4); for chronic feeding combined with EtOH binge gavage early in the morning: 4) 5% (vol/vol) EtOH for 10 days and binge feeding on day 11 with 5 g/kg body wt EtOH solution (EtOH 10d 1B, n = 8); 5) 5% (vol/vol) EtOH for 20 days combined with binge feeding on days 11 and 21 with 5 g/kg body wt EtOH solution (EtOH 20d 2B, n = 6); 6) 5% (vol/vol) EtOH for 40 days combined with binge feeding on days 11, 21, 31, and 41 with 5 g/kg body wt EtOH solution (EtOH 40d 4B, n = 6); and 7) pair-feeding: control mice were isocalorically pair-fed with control Lieber-DeCarli (Bio-Serv) diet for 10, 20, and 40 days. Half of the control animals with isocalorical maltose-dextrin (9 g/kg body wt) gavage served as controls for EtOH binge groups. Because we found no significant differences in the hemodynamic parameters of the pair-fed groups, we combined the animals into one pair-fed group (n = 7). An additional set of animals (without chronic EtOH feeding) was used to investigate the time course of blood alcohol levels at different time points (baseline, 1 h, 3 h, 6 h, and 9 h; n = 4 at each time point) after 5 g/kg body wt EtOH oral gavage. To exclude acute, direct effects of EtOH, we decided to perform all our in vivo experiments and sampling after 9 h of EtOH gavage, where EtOH levels returned near to the baseline values (Fig. 1B).

Echocardiography. At the end of the feeding period, depending on the feeding protocol, echocardiography was performed 9 h after the last EtOH or maltose-dextrin gavage. Mice were anesthetized with 1% to 2% isoflurane in 100% oxygen, placed on a temperature controlled heating pad, shaved and prepared for echocardiographic examination. Echocardiography was conducted by using a Vevo-770 Imaging system (FUJIFILM VisualSonics, Toronto, ON, Canada) coupled with RMV-707B (30 MHz) scanner, as described previously (14). M-mode images were taken on short-axis plane at the midpapillary level. B-mode images, acquired in the long-axis and in the short-axis at the midpapillary level, were used to measure left ventricular (LV) anterior wall (AW) and posterior wall (PW) thickness and LV internal diameter (ID) in end diastole (d) and in end systole (s). Pulse-wave Doppler measurements were performed to assess the ratio of the early (E) to late (A) ventricular filling velocities (E/A ratio). End systole was defined at minimal, whereas end diastole was defined at maximal, LVID. Values presented here were averages of three consecutive cycles. Fractional shortening (FS) was determined as FS = [(LVIdd − LVIdt)/LVIdd] × 100. LV mass was calculated by the following equation: LVMass = 1.04 × [(LVAWd + LVIdt + LVPWD)3 − LVIdt3] (6). LV volume was estimated according to the Teichholz formula (9). Ejection fraction (EF) was defined as the ratio of stroke volume and end-diastolic volume. Relative wall thickness (RWT) was calculated as RWT = (LVAWd + LVPWD)/LVIdt (23).

Hemodynamic measurements. After the echocardiographic examination, invasive hemodynamic measurements were performed by a pressure-conductance catheter method (MPV-US Ultra; Millar Instruments, Houston, TX) and a PVR-1045 (1F) pressure-volume (P-V) microcatheter (Millar Instruments) to assess detailed LV and vascular performance as described earlier (33). Mice were anesthetized with 1% to 2% isoflurane in 100% oxygen and placed on a temperature controlled heating pad. Hemodynamic parameters were analyzed by the PVAN software (Millar Instruments). Mean arterial pressure (MAP), heart rate, EF, mechanical efficiency (efficiency; the ratio of stroke work and P-V area), maximal slope of systolic pressure increment (dP/dtmax) and decrement (dP/dtmin), time constant of LV pressure decay (Taua; Weiss method), LV end-diastolic pressure (LVEDP), and arterial elastance (Ea) were analyzed. The slope (Eeva) of the LV end-systolic P-V relationships (ESPVR; linear model), the preload recruitable stroke work (PRSW), and the dP/dtmax-end-diastolic volume (EDV) were evaluated as pre- and afterload-independend systolic indexes, and the slope of the end-diastolic P-V relationship (EDPVR) was calculated as LV stiffness parameter. Ventriculo-arterial coupling was determined as the ratio of Ea and Eeva. Total peripheral resistance (TPR) was expressed as the ratio of MAP and cardiac output. At the end of the hemodynamic measurements, animals were euthanized and fresh frozen (in liquid nitrogen) LV samples were collected and stored at −80°C for further experimentation. For cryosectioning and histological staining, LV samples were fixed on dry ice in optimal cutting temperature compound (Tissue-Tek; Fisher Scientific, Pittsburgh, PA) and stored at −80°C for long term. For histological purposes, LV samples were fixed in 10% neutral buffered formalin and embedded in paraffin.

RT and real-time PCR analysis. LV samples were homogenized and total RNA was isolated by using QIAzol reagents (Qiagen, AJP-Heart Circ Physiol • doi:10.1152/ajpheart.00214.2016 • www.ajpheart.org
Table 1. Primer sequences used in quantitative RT-PCR experiments

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>P0, large, ribosomal protein</td>
<td>TCGAGGCCTGGGGCATTGA</td>
<td>TCTTATCCAGCTGAGATCAGTGAC</td>
</tr>
<tr>
<td>Atrial natriuretic peptide</td>
<td>TTTCCAGAGAACCTGCTAGGACCACTC</td>
<td>GCCAGGGCCTGAGTTTCTGCTT</td>
</tr>
<tr>
<td>p47phox</td>
<td>TCGCATCCCCTGGAAATGGAAAG</td>
<td>TCGCATGGCCTTAAACCGGAG</td>
</tr>
<tr>
<td>gp91phox (NOX2)</td>
<td>ATCCACAAAGGTTGCTACCCA</td>
<td>TGAATAGCCCCCTGCTGCA</td>
</tr>
<tr>
<td>Peroxisome proliferator-activated receptor α</td>
<td>AGACAGGGTGCTCCATGTTGCA</td>
<td>AGCAGACACACTTGATGCTCA</td>
</tr>
<tr>
<td>Peroxisome proliferator-activated receptor α</td>
<td>TGGGCCGTGCTTCTAAACATAG</td>
<td>TCTTGCAACAGTGGTGCAAGC</td>
</tr>
<tr>
<td>Medium-chain acyl-CoA dehydrogenase</td>
<td>GATGCAAGGTGCTCTGTTGACA</td>
<td>AGCTGATGCGCTATTCCAGAAA</td>
</tr>
<tr>
<td>Estrogen-related receptor α</td>
<td>ACCGCGCATCGGTGCTCTGCTC</td>
<td>GAGACGCGCGAGATAACCCA</td>
</tr>
<tr>
<td>Acetyl-CoA oxidase 1</td>
<td>TGGAGAGCCCCACACAAGGGAAG</td>
<td>CGATGCGCAGTTTGCAAA</td>
</tr>
<tr>
<td>Acetyl-CoA carboxylase 1</td>
<td>ACCGCGCGATGTTTGCACT</td>
<td>GCTTTCGCGCTCCTGCAAA</td>
</tr>
<tr>
<td>Collagen 1a</td>
<td>GACGGTGGGATGCTGGTGGC</td>
<td>CGTGGTAGTGGTAAAGCAGGAC</td>
</tr>
<tr>
<td>Fibronectin 1</td>
<td>AGAGACAGATGAGCTCCCTGCA</td>
<td>CGTGGTAGTGGTAAAGCAGGAC</td>
</tr>
</tbody>
</table>

The table shows the primer sequences for the target genes shown.
The effect of chronic alcoholism and binge drinking on body weight and heart weight. We did not observe any differences in the body weight, tibia length, and heart weight-to-tibia length ratio at the time of euthanization (Table 2). However, heart weight-to-body weight ratio was statistically different in EtOH 10d 1B from its corresponding chronic EtOH-fed group (Table 2).

**Hemodynamic alterations: cardiac dysfunction in chronic alcoholism and binge drinking.** Invasive hemodynamic investigation revealed that the development of alcoholic cardiomyopathy was associated with overt contractile dysfunction (Figs. 2A and 3A) and impaired relaxation (Fig. 3B) of the LV. In comparison with the Pair-fed group, the pre- and afterload independent, sensitive parameters of LV contractility (Ees, PRSW and dP/dtmax-EDV) were already significantly impaired in the EtOH 10d 1B group, which phenomenon worsened by the repetitive EtOH binges and increased duration of alcohol feeding in EtOH 20d 2B and EtOH 40d 4B groups, respectively (Fig. 3A). We observed a similar pattern in the case of the classic systolic parameter dP/dtmax (Fig. 3A). Interestingly, Ees and PRSW were significantly decreased in the EtOH 40d group than in the pair-fed group (Fig. 3A). Additionally, EF showed significant decrease in only EtOH 40d and EtOH 40d 4B groups when compared with the pair-fed group (Fig. 3A). Cardiac efficiency markedly worsened in the chronic EtOH feeding groups EtOH 20d 2B, EtOH 40d, and EtOH 40d 4B (Fig. 3A). Contractile function showed markedly deterioration in the chronic feeding plus binge groups when compared with their corresponding chronic feeding groups (Fig. 3A).

Beside the significant worsening of the contractile function, alcoholic cardiomyopathy was associated with significant impairment of LV relaxation (Tau, dP/dtmin) in binge drinking combined EtOH-fed groups (EtOH 10d 1B, EtOH 20d 2B, EtOH 40d 4B). *P < 0.05 vs. baseline (in case of the time course study) or vs. Pair-fed in chronic EtOH-fed groups.

Table 2. Study group characteristics in alcoholic cardiomyopathy

<table>
<thead>
<tr>
<th>Variable</th>
<th>Pair-fed</th>
<th>EtOH 10d</th>
<th>EtOH 10d 1B</th>
<th>EtOH 20d</th>
<th>EtOH 20d 2B</th>
<th>EtOH 40d</th>
<th>EtOH 40d 4B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>26.3 ± 0.4</td>
<td>25.0 ± 0.07</td>
<td>26.1 ± 0.7</td>
<td>26.3 ± 0.08</td>
<td>26.6 ± 0.5</td>
<td>27.8 ± 0.6</td>
<td>26.4 ± 0.6</td>
</tr>
<tr>
<td>Heart weight/tibia length, mg/mm</td>
<td>6.5 ± 0.1</td>
<td>6.9 ± 0.02</td>
<td>6.4 ± 0.2</td>
<td>6.7 ± 0.5</td>
<td>6.2 ± 0.5</td>
<td>7.3 ± 0.1</td>
<td>6.6 ± 0.2</td>
</tr>
<tr>
<td>Heart weight/body weight, mg/g</td>
<td>5.0 ± 0.1</td>
<td>5.4 ± 0.2</td>
<td>4.6 ± 0.1#</td>
<td>4.8 ± 0.4</td>
<td>4.4 ± 0.3</td>
<td>4.9 ± 0.1</td>
<td>4.8 ± 0.1</td>
</tr>
<tr>
<td>Tibial length, mm</td>
<td>19.3 ± 0.3</td>
<td>18.8 ± 0.3</td>
<td>18.9 ± 0.1</td>
<td>18.9 ± 0.2</td>
<td>18.9 ± 0.3</td>
<td>19.0 ± 0.4</td>
<td>19.0 ± 0.3</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>599 ± 10</td>
<td>616 ± 7</td>
<td>490 ± 13#</td>
<td>608 ± 10</td>
<td>511 ± 18#</td>
<td>623 ± 12</td>
<td>504 ± 19#</td>
</tr>
</tbody>
</table>

Values are means ± SE. The table shows the basic characteristic of the study groups. Groups: Pair-fed (isoscalorically fed with Lieber-DeCarli control liquid diet), ethanol (EtOH) Lieber-DeCarli liquid diet containing 5% (vol/vol) EtOH for 10 days (EtOH 10d); for 20 days (EtOH 20d); for 40 days (EtOH 40d); liquid diet containing 5% (vol/vol) EtOH for 10 days and binge feeding on day 11 with 5 g/kg BW EtOH solution (EtOH 10d 1B); for 20 days combined with binge feeding on days 11 and 21 with 5 g/kg BW EtOH solution (EtOH 20d 2B); for 40 days combined with binge feeding on days 11, 21, 31, and 41 with 5 g/kg BW EtOH solution (EtOH 40d 4B). *P < 0.05 vs. Pair-fed; #P < 0.05 vs. corresponding chronic EtOH-fed group.
Fig. 2. Chronic alcohol feeding and binge drinking impairs cardiac performance. A: representative pressure-volume loops in the study groups. B: representative M-mode images taken of the short-axis view at the midpapillary level of the left ventricle in the study groups. Yellow lines represent anterior and posterior wall thickness in end systole (AWs and PWs) and in end diastole (AWd and PWd). Red lines represent internal diameter in end systole (IDs) and in end diastole (IDd).

Groups: Pair-fed (isocalorically fed with Lieber-DeCarli control liquid diet), ethanol (EtOH) Lieber-DeCarli liquid diet containing 5% (vol/vol) EtOH for 10 days (EtOH 10d); for 20 days (EtOH 20d); for 40 days (EtOH 40d); liquid diet containing 5% (vol/vol) EtOH for 10 days and binge feeding on day 11 with 5 g/kg BW EtOH solution (EtOH 10d 1B); for 20 days combined with binge feeding on days 11 and 21 with 5 g/kg BW EtOH solution (EtOH 20d 2B); for 40 days combined with binge feeding on days 11, 21, 31, and 41 with 5 g/kg BW EtOH solution (EtOH 40d 4B).
CHRONIC PLUS BINGE ETHANOL FEEDING-INDUCED CARDIOMYOPATHY

Fig. 3. Chronic alcohol feeding and binge drinking is associated with impaired cardiac function. Systolic indexes were derived from pressure-volume analysis of the study groups. The following indexes are shown: slope of end-systolic pressure-volume relationship (Ees), preload recruitable stroke work (PRSW), maximal slope of systolic pressure increment (dP/dtmax), dP/dtmax-end-diastolic volume (dP/dtmax-EDV), ejection fraction (EF), and efficiency. B: indexes of diastolic function: time constant of left ventricular pressure decay (Tauw, according to Weiss method) and maximal slope of systolic pressure decrement (dP/dtmin). Stiffness parameters: left ventricular end-diastolic pressure (LVEDP) and slope of end-diastolic pressure-volume relationship (EDPVR). Groups: Pair-fed (isocalorically fed with Lieber-DeCarli control liquid diet), ethanol (EtOH) Lieber-DeCarli liquid diet containing 5% (vol/vol) EtOH for 10 days (EtOH 10d); for 20 days (EtOH 20d); for 40 days (EtOH 40d); liquid diet containing 5% (vol/vol) EtOH for 10 days and binge feeding on days 11 with 5 g/kg BW EtOH solution (EtOH 10d 1B); for 20 days combined with binge feeding on days 11 and 21 with 5 g/kg BW EtOH solution (EtOH 20d 2B); for 40 days combined with binge feeding on days 11, 21, 31, and 41 with 5 g/kg BW EtOH solution (EtOH 40d 4B). *P < 0.05 vs. Pair-fed; #P < 0.05 vs. corresponding chronic EtOH-fed groups.

Regarding the vascular indexes, we observed decreased MAP values in the EtOH 10d 1B, EtOH 20d 2B, and EtOH 40d 4B groups (Fig. 4) compared with the Pair-fed animals. In relation to these changes, alcohol intake induced remarkable decrease of TPR in the binge-fed EtOH 10d 1B, EtOH 20d 2B, and EtOH 40d 4B groups (Fig. 4). Although Ea was significantly lower already in the EtOH 10d and in binge-combined EtOH 10d 1B, EtOH 20d 2B, and EtOH 40d 4B groups (Fig. 4), the ventriculo-arterial coupling ratio showed significant increase in EtOH 10d 1B, EtOH 20d 2B, EtOH 40d, and EtOH 40d 4B groups compared with the Pair-fed and chronic EtOH-fed groups, respectively (Fig. 4).

Despite the significant worsening of cardiac function detected by invasive hemodynamic investigation (Fig. 3A), echocardiographic examination did not show any differences in the EF and FS between the study groups (Table 3). However, echocardiography revealed marked alterations in cardiac morphology in alcoholic cardiomyopathy (Fig. 2B). LVAWd and LVAWs values were significantly increased in EtOH 40d and EtOH 40d 4B groups compared with the Pair-fed (Table 3). Additionally, LVAWs was significantly increased in every EtOH-fed groups (Table 3), whereas LVIdd and LVIds did not differ among the study groups (Table 3). LVPWd and LVPWs showed marked elevation in only the EtOH 10d 1B, EtOH 20d 2B, and EtOH 40d 4B groups (Table 3). According to these, we found significant increase in RWT in EtOH 40d and EtOH 40d 4B groups compared with Pair-fed groups (Table 3). Despite this, the diastolic function marker E/A ratio and the LVmass did not differ among the study groups (Table 3).

Alcoholic cardiomypathy in chronic alcoholism combined with binge drinking is associated with excessive oxidative/nitrative stress and mitochondrial dysfunction. Myocardial 3-nitrotyrosine content was significantly elevated already in the EtOH 10d 1B group and increased with the increment of EtOH feeding duration and the number of binges in our model (Fig. 5A). Furthermore, we found significant elevation of the myocardial gene expression values of gp91phox, p47phox, and angiotensin II receptor, type 1a in hearts of mice on alcohol diets (Fig. 5B).

Chronic alcoholism combined with single or multiple binges impairs mitochondrial function, biogenesis, and fatty acid metabolism. The marked oxidative/nitrative stress resulted in myocardial mitochondrial dysfunction in our model (Fig. 6A). It is noteworthy, that the mitochondrial complex I activity was significantly lower at an early time point in EtOH 10d 1B group and continued to decrease by duration of EtOH feeding and by the number of EtOH binges (Fig. 6A). However, mitochondrial complex II and IV activities were intact at early time points but attenuated in EtOH 20d 2B, EtOH 40d, and EtOH 40d 4B groups compared with Pair-fed and chronic EtOH-fed groups (Fig. 6A).

Beside the observed dysfunction of the mitochondrial complexes in the alcoholic cardiac tissue, we found significant differences among the regulators of mitochondrial biogenesis and metabolism. EtOH feeding combined with binge drinking...
Fig. 4. Chronic alcohol feeding and binge drinking induces vascular dysfunction. Vascular indexes: arterial elastance (Ea), mean arterial pressure (MAP), ventriculo-arterial coupling and total peripheral resistance. Groups: Pair-fed (isocalorically fed with Lieber-DeCarli control liquid diet), ethanol (EtOH) Lieber-DeCarli liquid diet containing 5% (vol/vol) EtOH for 10 days (EtOH 10d); for 20 days (EtOH 20d); for 40 days (EtOH 40d); liquid diet containing 5% (vol/vol) EtOH for 10 days and binge feeding on day 11 with 5 g/kg BW EtOH solution (EtOH 10d 1B); for 20 days combined with binge feeding on days 11 and 21 with 5 g/kg BW EtOH solution (EtOH 20d 2B); for 40 days (EtOH 40d 4B). *P < 0.05 vs. Pair-fed; #P < 0.05 vs. corresponding chronic EtOH-fed groups.

Table 3. Echocardiography parameters in alcoholic cardiomyopathy

<table>
<thead>
<tr>
<th>Variable</th>
<th>Pair-fed</th>
<th>EtOH 10d</th>
<th>EtOH 10d 1B</th>
<th>EtOH 20d</th>
<th>EtOH 20d 2B</th>
<th>EtOH 40d</th>
<th>EtOH 40d 4B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ejection fraction, %</td>
<td>60 ± 3</td>
<td>64 ± 3</td>
<td>64 ± 1</td>
<td>61 ± 2</td>
<td>63 ± 3</td>
<td>63 ± 3</td>
<td>62 ± 2</td>
</tr>
<tr>
<td>Fractional shortening, %</td>
<td>33.8 ± 2.0</td>
<td>36.5 ± 3.4</td>
<td>33.2 ± 0.7</td>
<td>29.9 ± 0.6</td>
<td>32.4 ± 1.6</td>
<td>30.6 ± 3.5</td>
<td>33.9 ± 0.5</td>
</tr>
<tr>
<td>LVAWd, mm</td>
<td>0.80 ± 0.04</td>
<td>0.89 ± 0.01</td>
<td>0.81 ± 0.03</td>
<td>0.81 ± 0.03</td>
<td>0.78 ± 0.03</td>
<td>0.97 ± 0.03*</td>
<td>0.93 ± 0.03*</td>
</tr>
<tr>
<td>LVAVs, ms</td>
<td>1.02 ± 0.03</td>
<td>1.26 ± 0.05*</td>
<td>1.19 ± 0.03*</td>
<td>1.20 ± 0.05*</td>
<td>1.19 ± 0.05*</td>
<td>1.19 ± 0.03*</td>
<td>1.38 ± 0.04*</td>
</tr>
<tr>
<td>LVIDd, mm</td>
<td>3.78 ± 0.07</td>
<td>3.44 ± 0.14</td>
<td>3.63 ± 0.12</td>
<td>3.72 ± 0.05</td>
<td>3.62 ± 0.07</td>
<td>3.58 ± 0.09</td>
<td>3.49 ± 0.09</td>
</tr>
<tr>
<td>LVIDd, mm</td>
<td>2.64 ± 0.12</td>
<td>2.25 ± 0.19</td>
<td>2.47 ± 0.09</td>
<td>2.61 ± 0.03</td>
<td>2.45 ± 0.08</td>
<td>2.48 ± 0.12</td>
<td>2.39 ± 0.04</td>
</tr>
<tr>
<td>LVVPw, mm</td>
<td>0.75 ± 0.02</td>
<td>0.78 ± 0.02</td>
<td>0.90 ± 0.04*</td>
<td>0.81 ± 0.04</td>
<td>0.93 ± 0.04*</td>
<td>0.81 ± 0.04</td>
<td>0.88 ± 0.04*</td>
</tr>
<tr>
<td>LVPWs, mm</td>
<td>1.00 ± 0.03</td>
<td>1.08 ± 0.03</td>
<td>1.18 ± 0.06*</td>
<td>1.11 ± 0.05</td>
<td>1.22 ± 0.09*</td>
<td>1.10 ± 0.04</td>
<td>1.17 ± 0.06*</td>
</tr>
<tr>
<td>E/A ratio</td>
<td>1.51 ± 0.07</td>
<td>1.54 ± 0.26</td>
<td>1.61 ± 0.16</td>
<td>1.57 ± 0.29</td>
<td>1.37 ± 0.07</td>
<td>1.43 ± 0.05</td>
<td>1.37 ± 0.08</td>
</tr>
<tr>
<td>LVmass, mg</td>
<td>106.3 ± 4.5</td>
<td>100.1 ± 5.5</td>
<td>105.7 ± 7.8</td>
<td>105.6 ± 6.4</td>
<td>105.0 ± 7.0</td>
<td>111.3 ± 7.3</td>
<td>105.9 ± 5.1</td>
</tr>
<tr>
<td>Relative wall thickness</td>
<td>0.41 ± 0.02</td>
<td>0.46 ± 0.02</td>
<td>0.46 ± 0.02</td>
<td>0.44 ± 0.01</td>
<td>0.46 ± 0.02</td>
<td>0.50 ± 0.02*</td>
<td>0.51 ± 0.02*</td>
</tr>
</tbody>
</table>

Values are means ± SE. The table shows the results of the following echocardiographic parameters in alcoholic cardiomyopathy: ejection fraction, fractional shortening, left ventricular (LV) anterior wall thickness in diastole (LVAWd), LVAVW thickness in systole (LVAVs), LV internal diameter in diastole (LVIDd), LVID in systole (LVIDs), LV posterior wall thickness in diastole (LVPWd), LVPW in systole (LVPWs), ratio of the early (E) to late (A) ventricular filling velocities (E/A ratio), LV mass, and relative wall thickness. Groups: Pair-fed (isocalorically fed with Lieber-DeCarli control liquid diet), ethanol (EtOH) Lieber-DeCarli liquid diet containing 5% (vol/vol) EtOH for 10 days (EtOH 10d); for 20 days (EtOH 20d); for 40 days (EtOH 40d); liquid diet containing 5% (vol/vol) EtOH for 10 days and binge feeding on day 11 with 5 g/kg body wt EtOH solution (EtOH 10d 1B); for 20 days combined with binge feeding on days 11 and 21 with 5 g/kg body wt EtOH solution (EtOH 20d 2B); for 40 days combined with binge feeding on days 11, 21, 31, and 41 with 5 g/kg BW EtOH solution (EtOH 40d 4B). *P < 0.05 vs. Pair-fed; #P < 0.05 vs. corresponding chronic EtOH-fed group.
cross-sectional area was significantly elevated in our study groups, except for short-term EtOH feeding (EtOH 10d) in comparison with the Pair-feeding animals (Fig. 8A). Consistently with this, the gene expression of the hypertrophy marker ANP was increased (Fig. 8A).

Chronic alcoholism and binge drinking was not associated with increased myocardial fibrosis. Picro-Sirius Red staining and fibrotic area measurements did not show any difference among the study groups. mRNA levels of fibronectin 1 and Col1a1 did not differ in our models from the Pair-fed group (data not shown). Chronic alcohol feeding significantly affected the survival rate. Although the mortality was 0% in EtOH 10d and EtOH 10d 1B, it increased up to 25% in the long-term EtOH-fed groups (data not shown).

**DISCUSSION**

Excessive alcohol consumption leads to the development of nonischemic dilated cardiomyopathy, called alcoholic cardiomyopathy (44, 49). Alcoholic cardiomyopathy is characterized by LV dilation and hypertrophy (13). The pathophysiology of the disease includes the presence of cardiac oxidative/nitrative stress, mitochondrial dysfunction and disturbance of mitochondrial biogenesis, myocardial hypertrophy, LV dysfunction, and remodeling (34). Alcoholism-associated mortality and morbidity is closely related to the deleterious effects of the combination of chronic with binge drinking in humans. However, there are limited studies investigating the effect of binge drinking on the myocardium. Therefore, new animal models that mimic the drinking pattern of the human are warranted (34). Bertola et al. (3) reported the mouse model of chronic plus binge ethanol feeding, which closely mimics the human conditions of chronic long-term alcoholism with occasional binge drinking.

Herein, we report that modified NIAAA chronic and binge alcohol feeding mouse models are associated with 1) severe cardiac dysfunction, 2) excessive oxidative-nitrative stress, 3) deterioration of the mitochondrial complex activity and biogenesis, and 4) cardiac steatosis.

Ethanol is metabolized into acetaldehyde by ADH or CYP2E1, and acetaldehyde is transported to the mitochondria for further degradation through aldehyde-dehydrogenase to acetate and finally to acetyl-CoA (55). During the metabolism of ethanol ROS are produced as by-products, which results in pronounced oxidative stress (43, 45, 51). ROS are highly reactive molecules that bind to different proteins and enzymes, thereby reducing their function and leading to cellular malfunctions (32). Superoxide is able to react with NO to form the reactive nitrogen species peroxynitrite, which in turn damages different cellular targets, including contractile proteins, enzymes, and the mitochondria by oxidation and nitration (32).

As a results of the oxidative/nitrative damage, high amount of intramitochondrial ROS is produced (47); thus a vicious cycle of ROS production develops. Consistent with the above investigations, we have found significantly increased myocardial oxidative and nitrative stress. Our experiments showed that the degree of oxidative/nitrative stress positively correlated with the time being on alcohol feeding diet and by the number of EtOH binges. In alcoholism, mitochondrial injury occurs due to the overproduction of ROS, which causes mitochondrial deterioration of the mitochondrial complex activity and bioenergetics (32). Ethanol is metabolized in the heart through the fatty acid (FA) ethyl ester synthase into FA ethyl ester (2) that are potentially toxic products. They interact with several intracellular pathways, including mitochondrial oxidative phosphorylation or ATP production, thus contributing to the deleterious effects of EtOH. We observed decreased mitochondrial complex activity (complex I, II, and IV) in our models. It is noteworthy that the complex I activity was decreased already in the 10 d + 1 binge model, whereas complex II and IV activities were attenuated by increasing the number of binges and the duration of EtOH feeding. PGC1α, the master regulator of mitochondrial biogenesis, is directly damaged by EtOH due to its toxic effects (46). PGC1α is involved in a wide range of mitochondrial pathways through the regulation of different factors including PPARs and ERRα playing a major role in the uptake of energy substrates, ATP production, or in the FA oxidation (48). Additionally, EtOH directly deteriorates the citrate cycle (29). In summary, EtOH consumption leads to the imbalance of energy substrates along with blunted mitochondrial biogenesis. In line with the above findings, we observed significant decrease of PGC1α gene expression in all EtOH-fed groups combined with EtOH binges. Furthermore, EtOH consumption was associated with reduced mRNA levels of different regulators of mitochondrial metabolism, suggesting a complex impairment of mitochondrial biogenesis pathways, energy production, and FA metabolism.

FAs are key energetic source of the myocardium that are transported to the mitochondria for β-oxidation or stored as TG. Alcoholic cardiomyopathy has been described to be asso-
associated with the formation of lipid droplets and fat accumulation in the myocardium (16, 44). Hu et al. (16) showed enhanced myocardial lipid accumulation and increased long chain FA uptake during chronic alcohol consumption using a model of 10%, 14%, or 18% EtOH in drinking water for 12 wk, which is somewhat different from the human drinking pattern/condition. In contrast, we found cardiomyocyte lipid accumulation (evidenced by Oil Red O histology) already at an earlier time point (EtOH 10d) in our study, which was markedly enhanced by alcohol binge and tended to be attenuated with increased EtOH feeding period. In parallel, we observed significant upregulation of myocardial ACC 1 and 2, suggesting upregulation of the TG synthesis pathway. Interestingly, despite the application of multiple binges and long-term alcohol feeding, cardiac steatosis was reduced in the 20 and 40 days models indicated by Oil Red O results. The most likely explanation for the observed phenomenon is an adaptive process, which would be interesting to explore in the future studies. It has been reported that excessive amount of adipocytes and steatosis in the myocardium is associated with different cardiovascular diseases (57).

Pathological remodeling of the heart in alcoholic cardiomyopathy is a well-known phenomenon. As part of a complex cardiac pathology myocardial hypertrophy, apoptosis and fibrosis have been described in alcoholic cardiomyopathy (7, 43, 56). However, there are controversies regarding the extent and type of myocardial remodeling (37, 53). These features are mostly dependent on the frequency and the amount of alcohol consumption. In our model, mild cardiomyocyte hypertrophy was presented as indicated by the increased wall thicknesses and RWT on echocardiographic examination and by the enlarged cardiomyocyte cross-sectional area in all EtOH-fed groups.

Fig. 6. Chronic and binge drinking impairs mitochondrial function and biogenesis. A: mitochondrial complex I, II, and IV activities are shown in the myocardium. B: gene expression values of the following members of mitochondrial biogenesis are shown: PGC1α, peroxisome proliferator-activated receptor alpha (PPARα), estrogen-related receptor alpha (ERRα), medium-chain acyl-CoA dehydrogenase (MCAD), acetyl-CoA oxidase 1 (ACOX1). Groups: Pair-fed (isocalorically fed with Lieber-DeCarli control liquid diet), ethanol (EtOH) Lieber-DeCarli liquid diet containing 5% (vol/vol) EtOH for 10 days (EtOH 10d); for 20 days (EtOH 20d); for 40 days (EtOH 40d); liquid diet containing 5% (vol/vol) EtOH for 10 days and binge feeding on day 11 with 5 g/kg BW EtOH solution (EtOH 10d 1B); for 20 days combined with binge feeding on days 11 and 21 with 5 g/kg BW EtOH solution (EtOH 20d 2B); for 40 days combined with binge feeding on days 11, 21, 31, and 41 with 5 g/kg BW EtOH solution (EtOH 40d 4B). *P < 0.05 vs. Pair-fed; #P < 0.05 vs. corresponding chronic EtOH-fed groups.
complex approach is needed to investigate the effects of ethanol consumption on the cardiovascular performance. Therefore, we performed invasive hemodynamic examination and P-V analysis combined with echocardiography to characterize our models. P-V analysis provides the opportunity to measure pre- and afterload and heart rate independent systolic, contractility parameters, specific diastolic, cardiac stiffness, and vascular markers (33).

We observed marked attenuation of systolic indexes dP/dt max, Ees, PRSW, and dP/dt max-EDV indicating LV contractile dysfunction in all groups of chronic ethanol consumption combined with EtOH binges. Interestingly, 40d EtOH feeding by itself showed diminished contractility. Additionally, EF was significantly lower in only the 40d EtOH groups (with or without multiple binges) due to the rightward shift of P-V loops. Beside the prominent systolic dysfunction, we observed the significant worsening of diastolic LV relaxation (as shown by increased Tau w and decreased dP/dt min) in the EtOH feeding groups combined with single or multiple EtOH binges. In contrast with this, LV stiffness (according to LVEDP and the slope of EDPVR) was not increased in any of the groups investigated. Our functional data are in accordance with the in vitro data suggesting that the marked myocardial oxidative/nitrative stress combined with mitochondrial dysfunction and impaired biogenesis leads to an energetic crisis resulting in the abovementioned impairment of cardiac contractility and diastolic relaxation. However, our model was not associated with cardiac fibrotic

Fig. 7. Chronic alcoholism and binge drinking leads to myocardial fat accumulation. A: gene expression values of acetyl-CoA carboxylase 1 and 2 (ACC1, ACC2) of the left ventricle. B: representative images of Oil Red O stained left ventricle sections with lipid droplets in the cardiomyocytes. Magnification: 400×. Scale bar: 50 μm. Groups: Pair-fed (isocalorically fed with Lieber-DeCarli control liquid diet), ethanol (EtOH) Lieber-DeCarli liquid diet containing 5% (vol/vol) EtOH for 10 days (EtOH 10d); for 20 days (EtOH 20d); for 40 days (EtOH 40d); liquid diet containing 5% (vol/vol) EtOH for 10 days and binge feeding on day 11 with 5 g/kg BW EtOH solution (EtOH 10d 1B); for 20 days combined with binge feeding on days 11 and 21 with 5 g/kg BW EtOH solution (EtOH 20d 2B); for 40 days combined with binge feeding on days 11, 21, 31, and 41 with 5 g/kg BW EtOH solution (EtOH 40d 4B). *P < 0.05 vs. Pair-fed; #P < 0.05 vs. corresponding chronic EtOH-fed groups.
remodeling, which is usually an underlying mechanism of cardiac stiffening in many diseases (28, 39).

In addition to the diminished cardiac performance by P-V approach, we found deleterious effect of EtOH consumption on the vascular function as indicated by the lower MAP, TPR, and Ea parameters in single or multiple binges groups. It is noteworthy, that the systolic EF, FS, and the diastolic E/A ratio markers were not different measured with echocardiography among the groups, which is probably a consequence of the observed vascular changes and vasodilation.
Importantly, according to our data, the limitation of cardiac ultrasound examination and the vascular effects of alcohol should be taken into account when performing echocardiographic analysis to study the cardiac effects of ethanol consumption. Echocardiographic measurements are dependent on loading conditions and often not reliable in diseases with significant vascular alterations. Therefore, we performed invasive hemodynamic examination coupled with P-V analysis (33). The indexes derived from P-V analysis are pre- and afterload independent.

We also used P-V analysis to determine different mechanoenergetic parameters, such as efficiency and ventriculo-arterial coupling. We used the ratio of $E_{es}$ and $E_a$ to determine ventriculo-arterial coupling ratio (42). Ventriculo-arterial coupling ratio increased significantly in long-term alcohol feeding (EtOH 40d) and in single or multiple binge groups (EtOH 10, 20, and 40 with binges) indicating an inappropriate matching between the LV and arterial system. The mechanical efficiency is related to $E_{es}$, and the P-V area thus linearly correlates with the total oxygen consumption of the myocardium (41). Long-term alcohol feeding was associated with diminished cardiac efficiency, suggesting the reduction of metabolic efficiency (most probably due to the observed mitochondrial disturbances and oxidative/nitrative stress) in our model. To our knowledge, this is the first study reporting a detailed hemodynamic characterization of mouse alcoholic cardiomyopathy models due to a chronic EtOH feeding plus single or multiple EtOH binges.

Collectively, we demonstrate that chronic and binge drinking is associated with enhanced myocardial oxidative/nitrative stress, deteriorated mitochondrial function and biogenesis, cardiomyocyte hypertrophy, and myocardial steatosis, leading to impaired cardiovascular performance and mechanoenergetics.

ACKNOWLEDGMENTS

We thank Dr. George Kunos, the Scientific Director of NIAAA, for support.

GRANTS

The recent work was supported by the Intramural Research Program of NIAAA/NIH (to P. Pacher). C. Matyas was supported by the scholarship of the Hungarian-American Enterprise Scholarship Fund/Council on International Educational Exchange. Z. V. Varga was supported by the Rosztoczy Foundation.

DISCLOSURES

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

AUTHOR CONTRIBUTIONS


REFERENCES

H1670

CHRONIC PLUS BINGE ETHANOL FEEDING-INDUCED CARDIOMYOPATHY

