Chronic Ethanol Administration Prevents Compensatory Cardiac Hypertrophy in Pressure Overload

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Abstract

Background—Alcohol is among the most commonly abused drugs worldwide and affects many organ systems, including the heart. Alcoholic cardiomyopathy is characterized by a dilated cardiac phenotype with extensive hypertrophy and extracellular matrix (ECM) remodeling. We have previously shown that chronic ethanol administration accelerates the progression to heart failure in a rat model of volume overload. However, the mechanism by which this decompensation occurs is unknown. For this study, we hypothesized that chronic ethanol administration would prevent compensatory hypertrophy and cardiac remodeling in a rodent model of pressure overload (PO).

Methods—Abdominal aortic constriction was used to create PO in 8 week old male Wistar rats. Alcohol administration was performed via chronic intermittent ethanol vapor inhalation for 2 weeks prior to surgery and for the duration of the 8 week study. Echocardiography measurements were taken to assess ventricular functional and structural changes.

Results—Pressure overload increased posterior wall thickness and the hypertrophic markers, atrial and B-type natriuretic peptides (ANP and BNP). With the added stressor of ethanol, wall thickness, ANP, and BNP decreased in PO animals. The combination of PO and ethanol resulted in increased wall stress compared to PO alone. PO also caused increased expression of collagen I and III, whereas ethanol alone only increased collagen III. The combined stresses of PO and ethanol led to an increase in collagen I expression, but collagen III did not change, resulting in an increased collagen I/III ratio in the PO rats treated with ethanol. Lastly, Notch1 expression was significantly increased only in the PO rats treated with ethanol.

Conclusions—Our data indicate that chronic ethanol may limit the cardiac hypertrophy induced by PO that may be associated with a Notch1 mechanism, resulting in increased wall stress and altered ECM profile.

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Conflict of Interest

NWG owns shares in Glauser Life Sciences, Inc., a company with interest in the development of treatments for anxiety and addiction disorders.

Introduction

Alcohol is among the most commonly abused drug worldwide, and as such, chronic alcohol abuse creates an enormous economic burden on the healthcare system (Hasin et al., 2007). Chronic alcohol abuse has many health implications that affect multiple organ systems, including the heart (Mouton et al., 2016a, Piano, 2002). Alcoholic cardiomyopathy (ACM) is a term used to describe a dilated cardiomyopathy that is associated with chronic alcohol consumption (Piano, 2002). We have previously shown that chronic alcohol administration accelerates pathological remodeling in male rats with volume overload (VO)-induced heart failure (Mouton et al., 2016b). In that study, we found that ethanol accelerates the transition from compensatory remodeling in response to VO to an adverse eccentric hypertrophy with increased wall stress. However, the effects of ethanol exposure on pressure overload (PO), which is associated with concentric hypertrophy, are not known. The purpose of the current study was to investigate the effects of chronic alcohol administration on the compensatory mechanisms associated with PO-induced hypertrophy.

Heart failure occurs in response to sustained adverse stimuli, including pressure or volume overload, hypertension, diabetes, and chronic alcohol consumption (Kemp and Conte, 2012, Piano, 2002). Several studies have shown that cardiac hypertrophy and remodeling of the extracellular matrix (ECM) serve as compensatory mechanisms to normalize the increased wall stress due to adverse pressure and volume changes in the heart (Frohlich and Susic, 2012, Hutchinson et al., 2010). The progression of remodeling in response to sustained PO occurs in 3 stages: acute stimulus, compensatory remodeling, and decompensated heart failure (Piano, 2002). During decompensated failure, the heart shifts to a dilated phenotype called dilated cardiomyopathy (DCM) (Lakdawala et al., 2013). As many as two thirds of all DCM in the United States are associated with chronic ethanol abuse; however the mechanism by which ethanol accelerates transition into DCM remains unknown (Piano, 2002).

Notch1 is a transmembrane receptor that is well characterized in cardiac development and malignant tumors (Koenig et al., 2016, Timmerman et al., 2004, Bolos et al., 2007, Grego-Bessa et al., 2007). However, in recent years, Notch1 is described in adult cardiac tissue as a compensatory signaling mechanism (Croquelois et al., 2008, Gude and Sussman, 2012, Yang et al., 2016). A study by Croquelois et al. using a model of hypertension found that upregulation of Notch1 signaling limits cardiac hypertrophy (Croquelois et al., 2008). Further, studies show that ethanol has a significant impact on Notch1 signaling (Wang et al., 2014, Morrow et al., 2008, Morrow et al., 2010, Boopathy et al., 2013). Interestingly, the impact of ethanol on Notch1 signaling seems to be dependent on cell type. Morrow et al. found that ethanol decreases Notch1 signaling in human coronary artery smooth muscle cells, whereas ethanol stimulates signaling in human umbilical vein endothelial cells (Morrow et al., 2008, Morrow et al., 2010). In addition to its role in the hypertrophic response, Notch1 signaling is involved in epithelial-to-mesenchymal transition and enhances fibroblast activity (Kavian et al., 2012, Liu et al., 2009, Noseda et al., 2006, Timmerman et al., 2004). This role of Notch1 signaling could produce increased collagen production and contribute to fibrosis in the pressure overloaded heart (Kalluri and Neilson, 2003, Russell et al., 2011). Based on these findings, we hypothesized that ethanol increases cardiac Notch1

signaling, which impairs compensatory hypertrophy and produces fibrosis in response to PO, accelerating the transition to eccentric remodeling and failure.

Materials and Methods

Animal Groups: Dual-hit Model of Pressure Overload and Ethanol Administration

All experimental procedures were performed using 8 to 9 week-old male Wistar rats (~250 g). All studies conformed to the principles of the US National Institutes of Health *Guide for the Care and Use of Laboratory Animals 8th edition*, and were approved by LSU Health Sciences Center's Institutional Animal Care and Use Committee. Rats were randomized and divided into four groups: Sham-operated (Sham, n = 4), Sham + ethanol (EtOH, n = 3), untreated pressure Overload (PO, n = 3), and pressure Overload + ethanol (PO EtOH, n = 6). The cardiac effects of alcohol and pressure overload have been previously studied as independent stressors, but have not been studied in combination (Lang et al., 2005, Djousse and Gaziano, 2008, Peterson, 2002). As such, we used a smaller number of animals for the Sham, Sham + EtOH, and PO groups. As the results indicate, this low sample number provided sufficient statistical power for our endpoint measures. However, the low number of animals may limit interpretation of the data. For the ethanol-treated groups, rats were housed in ethanol vapor chambers for the entire duration of the study (La Jolla Alcohol Research, Inc. San Diego, CA), and exposed to a chronic intermittent ethanol vapor inhalation (14 hours on/10 hours off). Ethanol vapor levels were set to achieve blood alcohol levels (BALs) of 150–200 mg/dl. We have previously shown that chronic ethanol administration at this BAL produced end organ damage including markers of hepatic injury and inflammation, decreases in left ventricular wall thickness, increases in systolic blood pressure, and pulmonary inflammatory cell infiltration (Mouton et al., 2016a). BALs were assessed from tail vein blood samples using an Analox GM7 analyzer (London, UK). Rats were exposed to ethanol for a total of 8 weeks: 2 weeks of pretreatment before PO surgery followed by 6 additional weeks post-surgery. Food and water were provided ad libitum.

Pressure Overload Model: Abdominal Aortic Constriction

Rats were anesthetized using 3–3.5% isoflurane, and PO was surgically induced using abdominal aortic constriction. A laparotomy was performed, and the abdominal wall retracted. Blunt dissection was used to reveal the right and left renal arteries branching from the abdominal aorta. The abdominal aorta was partially constricted with a size-0 silk suture tied around a 21-gauge needle at the site between the branching of the left and right renal arteries (Nagalla et al., 2012). The needle was withdrawn leaving a patent, but constricted aorta. Sham animals underwent the same procedure with the exception of the ligation of the abdominal aorta.

Echocardiography

Left ventricular chamber dimension and function were assessed via echocardiography in sedated animals (1% isoflurane) (VEVO 770, VisualSonics; Toronto, CA). Cardiac functional measures were collected when BALs were 0 mg/dl. B-mode two-dimensional images and M-mode tracings of the LV posterior and anterior walls were obtained using LV

short-axis view. Using this method, LV end-diastolic diameter (LVEDD), and posterior and anterior wall thickness (LVPW and LVAW, respectively) at diastole (D) were measured.

Hydroxyproline Assay of Total Ventricular Collagen Concentration

Hydroxyproline assay was performed as previously described (El Hajj et al., 2016). Briefly, 40 mg of LV tissue was dried in an oven at 65° for 24 hours. Dried samples were then hydrolyzed in 6N HCl (10 mg dry weight to 1 ml HCl) at 120° for another 24 hours. Samples were then decolorized and filtered using activated charcoal, and dried by vacuum rotary evaporator. Hydroxyproline concentration was determined using the method described by Stegemann (Stegemann, 1958).

Western Blot Analysis

Western blot analyses were conducted as previously described (El Hajj et al., 2016). Briefly, 30 mg of LV free wall tissue for each sample was homogenized in RIPA buffer (#899900; Pierce Antibody Products; ThermoFisher Scientific; Waltham, MA) with HALT protease and phosphatase inhibitor cocktail (#78440; ThermoFisher Scientific). Bradford assay was used to assess protein concentration of homogenized tissue. Protein (50 µg) were subjected to SDS-PAGE and transferred to polyvinylidene fluoride membranes (PVDF). Indicated dilutions of primary antibody were incubated with PVDF membranes overnight at 4° against collagen I (1:1000, Product #34710; Abcam; Cambridge, UK), collagen III (1:5000, Abcam #7778), $\text{A}\alpha 1$ (1:50, Abcam #12104), HEY2 (1:400, Abcam #25404), and Notch1 (1:100, Abcam #52627). Histone H3 (1:5000, Abcam #1791) was used as a loading control. The membranes were incubated with secondary antibody (1:1000, Abcam #97051) for 1 hour at room temperature. Membranes were visualized by exposure to Western ECL substrate kit (Clarity, Biorad; Hercules, CA) and images collected with ImageQuant LAS 4000 imager (GE Healthcare Life Sciences; Marlborough, MA). Densitometry was performed using CareStream Software. All densitized bands were normalized to histone H3 expression.

Quantitative Real-time PCR

RNeasy Fibrous Tissue Kit (Qiagen; Hilden, Germany) was used to extract RNA from each LV sample. 1 µg of RNA from each sample was reverse transcribed into cDNA using iScript from Bio-Rad. qPCR was performed using SYBR green master mix (Bio-Rad #172-5121) with primers against COL1A1, COL3A1, Acta2, Myh6, Myh7, α -cardiac actin, and RPS13 (Integrated DNA Technologies; Coralville, IA). Additionally, primers against ANP and BNP were used (Sigma-Aldrich; St. Louis, MO). Thermocycling was performed using CFX96 Thermocycler (Bio-RAD), and data were analyzed using the C_T method. Gene expression for all genes was normalized to RPS13 values.

Statistics

All data were processed and analyzed using Graphpad (Prism; La Jolla, CA). Comparisons between each group were made using one-way analysis of variance (ANOVA) with Tukey's post-hoc analysis. Main effect and interactions between PO and ethanol treatment were determined with two-way ANOVA. A p-value of < 0.05 was considered significant. All values are shown as mean $\pm$ SEM, except for weights, which are reported as mean $\pm$ SD.

Results

Morphological Measurements

At the experimental endpoint body weights were measured. Additionally, kidney, liver, lungs, LV, and RV were weighed. Weights were normalized to tibial length (Fig 1). Tibial length alone did not change between any groups. Ethanol alone increased lung weight (Fig 1C), kidney weight (Fig 1D), and body weight (Fig 1F). After 8 weeks of PO, only LV weight was increased compared to Sham (Fig 1A). A combination of PO and EtOH significantly increased LV weight (Fig 1A), lung weight (Fig 1C), and kidney weight (Fig 1D) compared to Sham. Increases in lung weight are primarily indicative of pulmonary edema. The dramatic increase in kidney weight in the PO+EtOH group indicates a significant interaction between the PO and EtOH stressors (Fig 1D). There were no significant changes in RV or liver mass between any of the groups.

EtOH Impaired Compensatory Increases in Wall Thickness in PO Hearts

Cardiac function was noninvasively measured via echocardiography. Both fractional shortening and ejection fraction were preserved between all groups with an average of $41 \pm 2\%$ and $69 \pm 2\%$, respectively. Therefore, all animals were considered to be in the compensatory stage of cardiac remodeling. LV chamber size at end-diastole (LVEDD), and LV anterior and posterior wall thickness at diastole (LVAWD, LVPWD) were measured. These parameters indicate LV dilation and hypertrophy, respectively. There were no significant changes in anterior wall thickness between any of the groups (Fig 2A). Relative wall thickness was calculated using the equation: $2 \times \text{LVPWD} / \text{LVEDD}$. PO significantly increased posterior wall thickness (Fig 2B) and relative wall thickness (Fig 2D). However, the combination of PO and EtOH decreased relative wall thickness compared to PO alone (Fig 2D). This finding suggests that EtOH decreased compensatory wall thickening in the pressure overloaded heart.

EtOH Induced a Fetal-like Shift in the Myosin Heavy Chain Isoform and Disrupted Compensatory Hypertrophy in the PO Heart

Various markers associated with ventricular hypertrophy and sarcomeric remodeling were assessed using qPCR. Atrial natriuretic peptide (ANP) and b-type natriuretic peptide (BNP) are common markers of hypertrophy, whereas myosin heavy chain (Myh) 6 and 7 are markers of sarcomeric remodeling. Ethanol decreased Myh6 expression in PO hearts compared to both Sham and untreated PO (Fig 3); ethanol alone did not change Myh6 expression compared to Sham. PO increased Myh7 expression compared to Sham control and EtOH alone. Myh6/Myh7 ratio was calculated, wherein a decrease in the ratio indicates pathological fetal reprogramming of the myosin heavy chain isoform. The ratio decreased in both PO and PO EtOH hearts. Next, LV ANP and BNP were measured as markers of cardiac hypertrophy (Fig 3D and 3E). PO alone significantly increased ANP; however, ethanol decreased ANP expression in PO hearts. A similar trend was found with cardiac BNP expression. Pressure overload requires compensatory hypertrophy in order to normalize the wall stress imposed on the ventricles. We interpret the decrease in hypertrophic markers in the dual hit animals as ethanol blocking the compensatory remodeling during pressure overload. Further, α -cardiac actin mRNA expression was measured, as it is a primary

component of thin filaments in cardiac sarcomeres (Fig 3F). PO caused a significant increase in α -cardiac actin, but ethanol decreased expression in PO hearts.

Ethanol Exacerbated Fibrosis in the Pressure Overloaded Heart

Collagen protein expression was analyzed to assess whether chronic ethanol affected ECM remodeling in the pressure overloaded heart (Figure 4). Collagen I protein significantly increased in the PO group compared to both Sham and EtOH groups. Collagen I contributes tensile strength to the ECM and makes the heart more rigid and stiff. Ethanol alone did not change collagen I expression compared to Sham. Ethanol in combination with PO did not produce further increases in collagen I expression as compared to PO alone (Fig 4A), but was significantly elevated compared to Sham and EtOH alone. Collagen III expression was not significantly different from Sham levels in either the EtOH or PO alone groups. Collagen III produces a compliant phenotype in the ECM. Only the combination of PO and ethanol induced a significant decrease in collagen III in the heart compared to EtOH alone (Figure 4B). The ratio between collagen I to III was calculated. An increase in the collagen I/III ratio indicates a more fibrotic and stiffer matrix due to the relative prevalence of collagen I fibers. Ethanol alone produced a decreasing trend in the collagen I/III ratio compared to Sham controls. Though not statistically significant, a decrease in this ratio would indicate a more compliant ventricle (Fig 4C). Confirming the results of others, PO surgery alone increased the collagen I/III ratio (Kuwahara et al., 2002, Robert et al., 1994). However, the combination of PO and ethanol produced a significantly higher collagen I/III ratio compared to the PO, Sham, and EtOH groups. These data indicate that ethanol exacerbated the collagen remodeling in response to PO, producing a fibrotic and potentially stiffer ECM. To further confirm our collagen protein expression findings, a hydroxyproline (HPro) assay was performed in order to determine total collagen content (Fig 4D). The combined stresses of chronic ethanol and PO produced higher LV collagen content compared to Sham than either ethanol or PO alone. There were no Hpro differences from Sham control in either the EtOH or PO group.

Ethanol Increased Notch1 Signaling in the Pressure Overloaded Heart

Protein expression of Notch1 signaling components were measured, including Notch1 receptor, Aph1a, and Hey2 (Figure 5). Aph1a is a component of the gamma secretase complex that cleaves the Notch1 intracellular domain and Hey2 is a Notch1 transcription factor. While there were no significant changes in Notch1 expression in response to ethanol or PO alone, the combination of PO and ethanol significantly increased the protein expression of Notch1, Aph1a, and Hey2 (Fig 5) compared to Sham. The Hey2 protein expression increases in the PO EtOH group was significantly greater than the EtOH or PO group (Fig 5C).

Combined Ethanol and PO Adversely Affected Mediators of Oxidative Stress and Protein Synthesis

The metabolism of EtOH generates oxidative stress and has been shown to upregulate NADPH oxidase to further contribute to an increase in cellular oxidative stress. Oxidative stress affects intracellular signaling such as the balance between phosphatases as well as tyrosine kinases, which may contribute to the alterations in collagen that were found in

response to dual PO and ethanol. We measured NADPH oxidase 4 (NOX4), a key enzymatic contributor to cardiac oxidative stress, and nuclear factor erythroid 2–related factor 2 (Nrf2), a transcription factor for antioxidant enzymes. NOX4 is a major contributor to oxidative stress in the heart. Ethanol induced an increase in the enzyme NOX4 in PO hearts (Fig 6A). Both ethanol and PO stresses alone exhibited an increasing trend in Nrf2 expression; however, when combined, they significantly decreased Nrf2 expression, suggesting a decreased antioxidant capacity (Fig 6B)..

Discussion

Alcoholic cardiomyopathy is one of the most common causes of cardiac dilation (Zhang and Ren, 2011). It has been reported that alcoholic patients with cardiac structural and functional changes consume upwards of 90–200 grams of alcohol per day for 5–15 years (Fauchier et al., 2000, McKenna et al., 1998). This level of consumption can produce blood alcohol levels ranging from 120–200 mg/dL. The model of chronic ethanol administration used in our study produced comparable BALs. It should be noted that this duration and level of intoxication is representative of the most extreme cases of alcohol use disorder. The purpose of our study was to evaluate the adverse effects of chronic ethanol abuse on the cardiac compensatory response to pressure overload.

Cardiac remodeling due to a sustained adverse stimulus occurs in three stages: acute stimulus, compensatory remodeling, and decompensated heart failure (Hutchinson et al., 2010). This study focused on the compensatory remodeling stage of PO. In response to sustained PO, the LV undergoes concentric hypertrophy and remodeling of the ECM in order to alleviate wall stress caused by increased afterload (Hutchinson et al., 2010). However, this compensation is limited and the heart eventually transitions into decompensated failure. At this stage, the LV is no longer able to undergo concentric hypertrophy, and results in dilated cardiomyopathy (DCM). Since two-thirds of DCM cases in the U.S. are associated with alcohol abuse, we sought to examine if ethanol accelerates the progression of pressure overload induced remodeling. Using a rodent model of pressure overload, we investigated whether ethanol blocked compensatory hypertrophy in the pressure overloaded heart via Notch1 upregulation.

During compensatory remodeling, the heart undergoes concentric hypertrophy in which the myocytes synthesize sarcomeres in parallel with one another (Breisch et al., 1984, Norton et al., 2002). This type of hypertrophy thickens the walls of the heart in relation to interior chamber diameter in order to compensate for the increases in pressure. According to La Place's Law, ventricular wall stress is directly related to chamber diameter and pressure, and inversely related to ventricular wall thickness (Lorell and Carabello, 2000, Drazner, 2011). Thus, a compensatory increase in wall thickness in relation to the chamber diameter normalizes wall stress and prevents cardiac rupture (James et al., 1995). We observed increases in ventricular wall thickness in the pressure overload animals. However, when these animals were also exposed to chronic ethanol, the wall thickness decreased, indicating an increase in ventricular wall stress in these animals.

The myosin composition in the sarcomeres of the cardiomyocytes usually consists of α -myosin heavy chains, which is an adult isoform. However, as the heart transitions from the compensated stage of remodeling to decompensated heart failure, the myocytes undergo fetal reprogramming (Cox and Marsh, 2014, Pandya et al., 2006). As a result, during disease progression adult cardiac myocytes express β -myosin heavy chains, which is the fetal isoform. We assessed this process by analyzing the gene expression of both α and β isoforms through measuring mRNA levels of Myh6 and Myh7, respectively, in the cardiac LV tissue. A decrease in Myh6/Myh7 would indicate fetal reprogramming. This ratio decreased in both PO animals and PO EtOH groups, potentially indicating a transition into decompensated remodeling (Fernandes et al., 2011). PO animals expressed increased levels of the hypertrophic markers, ANP and BNP. PO animals treated with ethanol had a significant reduction in these hypertrophic markers, indicating further that ethanol prevented compensatory hypertrophy during pressure overload stress on the heart. These results mirror findings from our previous study in which ethanol prevented compensatory hypertrophy in volume overload induced heart failure (Mouton et al., 2016b).

The cardiac ECM serves many roles, which include providing a scaffolding for the cardiomyocytes and regulating growth factors and associated signaling (Taipale and Keski-Oja, 1997, Schultz and Wysocki, 2009, Kim et al., 2000). Cardiomyocytes attach to the ECM via integrins that relay mechanical force to the muscle cells (Borg et al., 1983). Changes in ECM composition can significantly impair mechanotransduction and cardiomyocyte viability (Kim et al., 2000). Further, the ECM serves as a reservoir for growth factors such as TGF- β , fibroblast growth factor, as well as others (Taipale and Keski-Oja, 1997). Modulation of the ECM affects the signaling of these growth factors and can influence cardiomyocytes. The main composition of the cardiac ECM is collagen I and III fibers. Collagen I provides a more tensile and stiff phenotype, whereas collagen III gives a more compliant phenotype; therefore, an increase in collagen I/III ratio would indicate a stiffer ventricle. Consistent with previous studies, LV collagen I/III ratio was increased by pressure overload (Robert et al., 1994, Kuwahara et al., 2002). We assessed protein expression of both collagens I and III in whole LV tissue. Interestingly, we found that collagen I/III ratio increased dramatically in the PO EtOH hearts as compared to PO or ethanol alone. Collagen content was measured through a hydroxyproline assay, and the total collagen content increased the greatest in the PO EtOH hearts. Our results indicate that the dual-hit animals have a decrease in posterior wall thickness and an increase in chamber dilation, suggesting an increased wall stress.

We next sought to delineate possible mechanisms of how chronic ethanol prevented compensatory hypertrophy in the PO heart. Previous studies show that Notch1 activation is a key signaling mechanism involved in cardiac hypertrophy (Nemir et al., 2014, Croquelois et al., 2008). Notch1 is generally considered to be critical during normal cardiac development in embryos, but it is relatively quiescent in the healthy adult heart (Gude and Sussman, 2012). However, in response to pathophysiological stressors, Notch1 signaling is significantly activated (Croquelois et al., 2008). Croquelois et al. found that cardiac Notch1 is upregulated in hypertension using a one kidney, one clip model in mice. However, they suggested that Notch1 plays a role in limiting hypertrophy. Notch1 prevents the uncontrolled hypertrophic growth of cardiomyocytes during cardiac adaptation by inhibiting the

transcription of GATA4 (Kathiriyai et al., 2004). In our study, we found that Notch1 protein expression increased in the LV tissue of dual-hit PO EtOH animals, concomitant with a reduction in LV wall thickness. It is possible that ethanol dysregulates the compensatory upregulation of Notch1 signaling, thereby inhibiting the compensatory hypertrophic responses to PO. Notch1 inhibits the transcription of cardiac genes through transcriptional inhibition of GATA4, which includes inhibition of hypertrophic genes. In separate studies by Boopathy et al., ethanol activates Notch1 signaling through oxidative stress pathways (Boopathy et al., 2013). Additionally, the genes responsible for fibroblast activation are a direct target of Notch1 signaling (Nosedai et al., 2006). As expected, Notch1 receptor expression increased in response to ethanol, PO, and combined. The receptor is a transmembrane protein, with an extracellular and intracellular component. Upon receptor activation, the intracellular domain is enzymatically cleaved by λ -secretase (Kopan and Ilagan, 2009). Both Notch1 receptor and λ -secretase subunit Apha1a expression increased in the PO EtOH group. This finding would suggest increased activation, although the actual activity of the Notch intracellular domain was not assessed. Once the Notch1 intracellular domain is cleaved, it translocates into the nucleus where it activates the transcription factor c-promotor binding protein factor 1/suppressor of hairless/LAG1 (CSL) (Kopan and Ilagan, 2009). Among the downstream targets of CSL is the transcription factor Hey2, which was also upregulated in the PO EtOH animals. These data support our hypothesis that EtOH increases Notch1 signaling and activity through its downstream effectors.

Altogether, the cardiac adaptation to stressors such as pressure overload is cardiomyocyte hypertrophy, which was evident in our PO group through increased hypertrophic markers and posterior wall thickening. In PO animals treated with ethanol, both of these indicators were decreased despite the persistent pressure overload stress. Notch1 is thought to be cardioprotective by limiting hypertrophy during disease. However, it is possible that in the presence of ethanol, this cardioprotection is dysregulated. Overactivation of Notch1 may limit hypertrophy during pressure overload, where hypertrophy is a necessary compensatory response to maintain cardiac function. Indeed, we found that Notch1 was significantly upregulated and greatest in the hearts of the combined PO EtOH animals.

It is well accepted that chronic ethanol exposure creates an environment of oxidative stress in multiple organs, including the heart (Zhang et al., 2004). This oxidative injury occurs primarily through the metabolism of ethanol. We have previously shown that chronic ethanol administration increases NOX4, which is the major enzyme that creates oxidative stress in the heart. In this current study, we also show that NOX4 protein expression is upregulated in the PO EtOH hearts. Oxidative stress affects intracellular signaling such as the balance between phosphatases (PTEN and PP1/PP2) as well as tyrosine kinases (TGF- β receptor), which may contribute to the alterations in collagen that we found in response to dual PO and ethanol (Jiang et al., 2014). In addition to NOX4, we measured Nrf2. Nrf2 is a transcription factor that regulates antioxidant capacity within the cell. It transcribes genes for glutathione synthesis, superoxide dismutase, and other important antioxidant defenses (Hayes et al., 2000, Nguyen et al., 2009). We found that Nrf2 protein expression was upregulated by ethanol and PO alone, and we interpret these increases as cellular defense against these stressors. However, in the dual-hit PO EtOH animals, cardiac Nrf2 was significantly

decreased. This finding indicates a depletion of antioxidant defenses and an environment of increased oxidative stress within these hearts.

In conclusion, chronic ethanol exposure disrupted compensatory hypertrophy in the pressure overloaded hearts. This disruption was detrimental as it prevented the heart from adapting to the pressure overload stressor by limiting appropriate compensatory remodeling of the cardiac myocyte and ECM. Our data indicate that these ethanol-dependent effects on remodeling were associated with dysregulation of Notch1 signaling. These disruptions in Notch signaling may contribute to the switch from compensatory remodeling into decompensated heart failure found in alcoholic cardiomyopathy.

Acknowledgments

Funding: NIH NIAAA 1R21AA0226901 (Jason D. Gardner); Saving Tiny Hearts Society (Jason D. Gardner); NIH NIAAA T32AA007577 (Patricia E. Molina); NIH NHLBI 1F31HL134263 (Elia C. El Hajj)

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Figure 1.

Morphological measurements after 8 weeks. Pressure overload (PO) and the combination of PO and ethanol (PO EtOH) groups developed left ventricular (LV) hypertrophy (A) compared to sham controls (Sham). There were no significant differences in right ventricular weight (B) when normalized to tibia length between any groups. The combination of PO and EtOH as well as EtOH alone increased lung mass (C). EtOH alone and combination PO and EtOH increased right kidney weight compared to Sham (D). Combination of PO and EtOH increased right kidney weight versus PO alone. There were no differences in liver mass between any groups (E). EtOH treatment alone increased bodyweight (F). Values are mean $\pm$ SD (*p<0.05, **p<0.01, ***p<0.0001).

Figure 2.

Echocardiography measurements of left ventricular (LV) wall and chamber dimensions. Pressure overload (PO) increased posterior wall thickness and relative wall thickness versus Sham (B). There were no significant differences in anterior wall thickness between any groups (A). There were no significant changes in LV end diastolic diameter in any group (C). PO caused increased relative wall thickness compared to Sham; however, the combination of PO and ethanol (EtOH) decreased relative wall thickness compared to PO surgery alone. Values are mean $\pm$ SE (* p <0.05, ** p <0.01).

Figure 3.

Fetal reprogramming and hypertrophic gene expression. (A) Combination pressure overload (PO) and ethanol (EtOH) decreased Myh6 mRNA expression compared to PO alone. (B) PO increased Myh7 expression compared to both Sham and EtOH groups. (C) There were no significant differences in Myh6/Myh7 ratio, but there was a decreasing trend in PO and PO EtOH groups compared to Sham. (D) PO increased ANP compared to Sham and combination of PO and EtOH decreased ANP compared to PO alone. (E) Likewise, combination PO and EtOH decreased BNP expression compared to PO alone. (F) PO increased α-cardiac actin expression compared to Sham, but combination PO and EtOH was decreased compared to PO alone (* $p < 0.05$, ** $p < 0.01$).

Figure 4.

Collagen protein expression and myocardial collagen content. (A) Collagen I protein expression was increased by pressure overload (PO) as compared to Sham and ethanol (EtOH) groups. Collagen I also increased in PO EtOH compared to EtOH alone. (B) The combination of PO and EtOH decreased collagen III expression compared to EtOH alone. (C) Combination of PO and EtOH increased collagen I/III ratio compared to Sham, PO and EtOH. (D) Myocardial collagen content, assessed by hydroxyproline (HPro), was significantly increased only in the PO EtOH group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Figure 5.

Notch1 signaling pathway components. (A) The combination of pressure overload (PO) and ethanol (EtOH) increased Notch1 receptor protein expression compared to Sham controls. (B) Likewise, the combination of PO and EtOH increased gamma secretase subunit Aph1a protein expression compared to Sham controls. (C) Lastly, the combination of PO and EtOH also increased Hey2 transcription factor protein expression compared to EtOH or PO surgery alone (* $p < 0.05$).

Figure 6. Oxidative stress-related and protein synthesis mediators. (A) The combined stress of pressure overload (PO) and ethanol (EtOH) increased left ventricular (LV) NOX4 expression compared to Sham controls. (B) PO and EtOH caused an increased in Nrf2 expression which was blocked in the dual-hit PO EtOH group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).