Cardiac Overexpression of Alcohol Dehydrogenase Exacerbates Cardiac Contractile Dysfunction, Lipid Peroxidation, and Protein Damage After Chronic Ethanol Ingestion


Background: Alcoholic cardiomyopathy is manifested as ventricular dysfunction, although its specific toxic mechanism remains obscure. This study was designed to examine the impact of enhanced acetaldehyde exposure on cardiac function via cardiac-specific overexpression of alcohol dehydrogenase (ADH) after alcohol intake.

Methods: ADH transgenic and wild-type FVB mice were placed on a 4% alcohol or control diet for 8 weeks. Mechanical and intracellular Ca²⁺ properties were evaluated in cardiac myocytes. Levels of acetaldehyde, lipid peroxidation, and protein carbonyl formation were determined.

Results: FVB and ADH mice consuming ethanol exhibited elevated blood ethanol/acetaldehyde, cardiac acetaldehyde, and cardiac hypertrophy compared with non-ethanol-consuming mice. However, the levels of cardiac acetaldehyde and hypertrophy were significantly greater in ADH ethanol-fed mice than FVB ethanol-fed mice. ADH transgene itself did not affect mechanical and intracellular Ca²⁺ properties with the exception of reduced resting intracellular Ca²⁺ and Ca²⁺ re-sequestration at low pace frequency. Myocytes from ethanol-fed mice showed significantly depressed peak shortening, velocity of shortening/relengthening, rise of intracellular Ca²⁺ transients, and sarco(endo)plasmic reticulum Ca²⁺ load associated with similar duration of shortening/relengthening compared with myocytes from control mice. Strikingly, the ethanol-induced mechanical and intracellular Ca²⁺ defects were exacerbated in ADH myocytes compared with the FVB group except velocity of shortening/relengthening. The lipid peroxidation end products malondialdehyde and protein carbonyl formation were significantly elevated in both livers and hearts after chronic ethanol consumption, with the cardiac lipid and protein damage being exaggerated by ADH transgene.

Conclusion: These data suggest that increased cardiac acetaldehyde exposure due to ADH transgene may play an important role in cardiac contractile dysfunctions associated with lipid and protein damage after alcohol intake.

Key Words: Acetaldehyde, Ventricular Myocytes, Lipid Peroxidation, Protein Carbonyl, Shortening, Intracellular Ca²⁺ Transient.

Chronic alcoholism leads to heart muscle damage and onset of alcoholic cardiomyopathy, accounting for one third of dilated cardiomyopathy. The incidence of cardiomyopathy is nearly 50% in patients with chronic alcoholism, and most of these patients die from cardiac disease (Fernandez-Sola et al., 1994). Alcoholic cardiomyopathy is characterized by cardiomegaly, disruptions of myofibrillar architecture, reduced myocardial contractility, decreased ejection volumes, and enhanced risk of stroke and hypertension (Patel et al., 1997; Richardson et al., 1998). Although several hypotheses have been postulated for the pathogenesis of alcoholic cardiomyopathy including direct and indirect cardiotoxicity of alcohol (Preedy et al., 1999) and accumulation of fatty acid ethyl esters (Laposata and Lange, 1986), neither notion received convincing clinical and experimental support to be fully validated.

Acetaldehyde, the first oxidized metabolic product of ethanol, has been considered a candidate toxin for the onset of alcoholic cardiomyopathy because it concentrates in the heart and affects the ability to inhibit cardiac protein synthesis (Espinet and Argiles, 1984; Siddiq et al., 1993) and is far more reactive than ethanol. Our laboratories have shown that acetaldehyde may directly impair cardiac excitation-contraction (E-C) coupling and inhibit sarco(en-
do)plasmic reticulum (SR) Ca$^{2+}$ release function (Brown et al., 1999; Ren and Brown, 2000; Ren et al., 1997, 2002). However, advancement of the acetaldehyde toxicity theory was relatively limited over the past years due to lack of a suitable method to chronically alter acetaldehyde levels in vivo. Although blood acetaldehyde may reach low mM levels after alcohol intake in Asians and African Americans due to defective aldehyde dehydrogenase (ALDH) (Tsukamoto et al., 1989; Yoshida, 1992), intolerance among these populations to alcohol ingestion makes them practically impossible to be considered for the study of acetaldehyde. Earlier work with metabolic inhibitors to alter acetaldehyde levels revealed that the metabolic inhibitors for acetaldehyde are nonspecific, ineffective, toxic, and difficult to manage chronically (Preedy and Richardson, 1994). To overcome such obstacles in the assessment of acetaldehyde, we developed a transgenic mouse line to overexpress alcohol dehydrogenase (ADH) specifically in the heart, which elicited higher cardiac acetaldehyde levels associated with damaged whole heart function and morphology after chronic ethanol intake (Liang et al., 1999). We further showed exacerbated cardiac contractile depression in response to acute ethanol administration in isolated ventricular myocytes (Duan et al., 2002, 2003). However, whether chronically elevated acetaldehyde levels facilitate the onset of alcoholic cardiomyopathy directly through individual cardiac myocytes or indirectly through interstitial nonmyocyte components as well as the underlying mechanism of action is essentially unknown. The aim of this study was to investigate the impact of elevated cardiac acetaldehyde exposure via transgenic overexpression of ADH enzyme after alcohol intake on cell shortening, intracellular Ca$^{2+}$ homeostasis, and membrane lipid and protein damage. The latter was chosen largely due to the fact that chronic alcohol intake is known to enhance oxidative stress and damage of membrane lipid and protein (Preedy et al., 1999), although whether this damage is intimately linked to enhanced acetaldehyde level is unclear.

MATERIALS AND METHODS

Experimental Animals and Chronic Ethanol Ingestion

All animal procedures were approved by the University of North Dakota Institutional Animal Care and Use Committee. Briefly, ADH transgene was constructed and driven by the mouse α-myosin heavy chain promoter to achieve specific cardiac overexpression. The complementary DNA (cDNA) for murine class I ADH was inserted behind the promoter. This cDNA was chosen because class I ADH is the most efficient in the oxidation of ethanol. Wild-type FVB mice obtained from the University of North Dakota Biomedical Research Center were used to produce transgenic lines containing the ADH transgene (Taketo et al., 1991). A second transgene with a cDNA encoding tyrosinase was coinjected with ADH. This enzyme produces coat color pigmentation in albino mice and was used to conveniently identify transgenic animals. All mice were housed in a temperature-controlled room under a 12 hr/12 hr light/dark schedule and were allowed access to tap water ad libitum. Three to four-month-old adult male FVB and ADH mice were selected for study and were introduced to a nutritionally complete liquid diet (Shake & Pour Bioserv Inc., Frenchtown, NJ) for a 1 week acclimation period. The use of a liquid diet is based on the scenario that ethanol self-administration results in less nutritional deficiencies and less stress to the animals compared with forced-feeding regimens, intravenous administration, or aerosolized inhalation (Keane and Leonard, 1989). On completion of the acclimation period, half of the FVB and ADH mice were maintained on the regular liquid diet (without ethanol), and the remaining half began an 8 week period of isocaloric 4% (v/v) ethanol diet feeding. An isocaloric pair-feeding regimen was used to eliminate the possibility of nutritional deficits. Control mice were offered the same quantity of diet that ethanol-consuming mice drank the previous day. Body weight was monitored weekly (Ren and Brown, 2000).

Assessment of Ethanol and Acetaldehyde Levels

On the morning of the last day of chronic ethanol or control diet feeding, mice were killed under anesthesia, and blood, liver, and a piece of heart were collected in sealed vials and stored at −80°C. Immediately before analysis, the samples were warmed to 25°C. A 2 ml aliquot of the headspace gas from each vial was removed through the septum on the cap with a gas-tight syringe and transferred to a 200 μl loop injection system on a Hewlett-Packard 5890 gas chromatograph equipped with a flame ionization detector. Ethanol, acetaldehyde, and other components were separated on a 9 m VOCOL capillary column (Supelco Inc., Bellefonte, PA) with film of 1.8 μm thickness and an inner diameter of 0.32 mm. The temperature was held isothermally at 30°C, and the carrier gas was helium at a flow rate of 1.8 ml/min. Under these conditions, separation of acetaldehyde from ethanol and other compounds was complete in 1 min. Quantitation was achieved by calibrating the gas chromatograph peak areas against those from headspace samples of known ethanol and acetaldehyde standards, over a similar concentration range as the tissue samples in the same buffer (Duan et al., 2002, 2003).

Cell Isolation Procedures

Mouse hearts were removed and perfused with Krebs-Henseleit bicarbonate buffer containing (in mM) 118 NaCl, 4.7 KCl, 1.2 MgSO₄, 1.2 KH₂PO₄, 25 NaHCO₃, 10 HEPES, and 11.1 glucose, with 5% CO₂/95% O₂. Hearts subsequently were digested with 223 units/ml collagenase D (Boehringer Mannheim, Indianapolis, IN) for 20 min at 37°C. After perfusion, ventricles were removed and minced before being filtered. Extracellular Ca$^{2+}$ was slowly added back to 1.25 mM. Functional study was conducted within 6 hr of cell isolation, and myocytes with obvious sarcosomal blebs or spontaneous contractions were not used (Duan et al., 2002).

Cell Shortening/Relengthening

Mechanical properties of ventricular myocytes were assessed by using an IonOptix SoftEdge system (IonOptix, Milton, MA) as described (Duan et al., 2002). In brief, cells were placed in a chamber mounted on the stage of an inverted microscope (Olympus IX-70) and superfused (~2 ml/min at 37°C) with a buffer containing (in mM) 131 NaCl, 4 KCl, 1 CaCl₂, 1 MgCl₂, 10 mM glucose, and 10 HEPES, at pH 7.4. The cells were field stimulated to contract at a frequency of 0.5 Hz unless otherwise specified. Changes in cell length during shortening and relengthening were captured and converted to digital signal before being analyzed with IonWizard software. The myocyte being studied was scanned rapidly with a camera at 120 Hz to ensure recording with good fidelity. Cell shortening and relengthening were assessed by using the following indexes: peak shortening (PS), indicative of peak ventricular contractility; time to PS (TPS), indicative of systolic duration; time to 90% relengthening (TR90), indicative of diastolic duration; and maximal velocities of shortening/relengthening, indicative of maximal velocities of ventricular pressure increase/decrease. In the case of altering stimulus frequency, the steady-state contraction of myocyte was achieved (usually after the first five to six beats) before PS was recorded.
Measurement of Intracellular Ca\(^{2+}\) Transient and SR Ca\(^{2+}\) Load

Myocytes were loaded with fura-2/AM (0.5 \(\mu\)M) for 10 min at 30°C, and intracellular Ca\(^{2+}\) transients were recorded with a dual-excitation fluorescence photomultiplier system (IonOptix, Milton, MA) as described (Duan et al., 2002). Myocytes imaged through an Olympus IX-70 Fluor \(\times 40\) oil objective were exposed to light emitted by a 75 W lamp and passed through either a 360 or a 380 nm filter (bandwidths were \(\pm 15\) nm) while being stimulated to contract at 0.5 Hz. Fluorescence emissions were detected between 480 and 520 nm by a photomultiplier tube after first illuminating the cells at 360 nm for 0.5 sec and then at 380 nm for the duration of the recording protocol (333 Hz sampling rate). The 360 excitation scan was repeated at the end of the protocol, and qualitative changes in intracellular Ca\(^{2+}\) were inferred from the ratio of the fluorescence intensity at the two wavelengths. SR Ca\(^{2+}\) loading capacity was assessed by rapid puff of caffeine (10 mM)-induced intracellular Ca\(^{2+}\) transient intensity in fura-2-loaded ventricular myocytes. Caffeine triggers release of Ca\(^{2+}\) from SR, the major pool of Ca\(^{2+}\) available to contractile proteins in rodent cardiac muscle. Multiple applications of caffeine were given at 5 min intervals to ensure steady state (Ren et al., 2002).

Determination of Lipid Peroxidation End Product Malondialdehyde

Malondialdehyde (MDA) in livers and hearts was determined by high performance liquid chromatography. In brief, 0.5 ml of tissue suspension or a standard solution of 1,1,3,3-tetramethoxypropane (Sigma Chemical, St. Louis, MO) was treated with 50 \(\mu\)l of 0.2% (w/v) butylated hydroxytoluene (Sigma Chemical) in ethanol. The mixture was divided into two tubes (for duplicate determinations) to each of which was added 1.5 ml of toluene (Sigma Chemical) solution was added to the samples, which were heated gently to 60°C to dissolve the thiobarbituric acid. The samples then were heated to 80°C to produce MDA and the mixture was allowed to cool to room temperature for 10 min. The samples were centrifuged, and the supernatant was added to 0.5 ml of 0.6% (w/v) thiobarbituric acid (Sigma Chemical) solution was added to the samples, which were heated gently to 80°C to dissolve the thiobarbituric acid. The samples then were heated to 90°C for 45 min and cooled on ice. The mixture was centrifuged at 4°C at 6000 rpm for 5 min, and the supernatant was removed and filtered with a 0.2 \(\mu\)m Gelman PTFE syringe filter (Pall Life Sciences, Ann Arbor, MI). Samples were eluted with a Shimadzu model SCL 10-A VP high performance liquid chromatography system fitted with a Shimadzu SIL-10A VP HPLC pump (Shimadzu, Columbia, MD) and a Waters Symmetry column (C18, 5 \(\mu\)m particle size, 4.6 mm \(\times\) 250 mm; Waters, Milford, MA). Then 20 \(\mu\)l of filtered supernatant was injected into a mobile phase made up of 65% 50 mM KH\(_2\)PO\(_4\) (adjusted to pH 7.0 with 3 M KOH) and 35% methanol and run at a flow rate of 1 ml/min. The MDA peak of interest was eluted at 8 min and detected by using a Waters 474 scanning fluorescence detector operating at 532 nm excitation and 553 nm emission (Chirico, 1994).

Protein Carbonyl Assay

To assess oxidative protein damage in liver and hearts, the carbonyl content of protein extracted from tissues was determined as described (Levine et al., 1990; Ren et al., 2003). Briefly, proteins were extracted and minced to prevent proteolytic degradation. Nucleic acids were eliminated by treating the samples with 1% streptomycin sulfate for 15 min, followed by a 10 min centrifugation (11,000 \(\times\) g). Protein was precipitated by adding an equal volume of 20% TCA to protein (0.5 mg) and centrifuged for 1 min. The TCA solution was removed and the sample resuspended in 10 mM 2,4-dinitrophenylhydrazine solution. Samples were incubated at room temperature for 15 to 30 min. After 500 \(\mu\)l of 20% TCA was added, samples were centrifuged for 3 min. The supernatant was discarded and the pellet was washed in ethanol/ethyl acetate and allowed to incubate at room temperature for 10 min. The samples were centrifuged again for 3 min, and the ethanol/ethyl acetate steps were repeated two more times. The precipitate was resuspended in 6 M guanidine solution and centrifuged for 3 min, and insoluble debris was removed. The maximum absorbance (360–390 nm) of the supernatant was read against appropriate blanks (water, 2 M HCl), and the carbonyl content was calculated by using the molar absorption coefficient of 22,000 M\(^{-1}\)cm\(^{-1}\).

Data Analysis

For each experimental series, data are reported as mean \(\pm\) SEM. Differences between groups was assessed by using the Student’s \(t\) test, whereas within-group comparisons between mean values were calculated by repeated measures analysis of variance (ANOVA). When an overall significance was determined, a Dunnnett’s post hoc analysis was incorporated. We considered \(p < 0.05\) to be significant.

Table 1. General Feature of Wild-Type (FVB) and ADH Transgenic Mice After 8 Weeks of Ethanol (EtOH) or Control Diet

<table>
<thead>
<tr>
<th>Parameter</th>
<th>FVB</th>
<th>ADH</th>
<th>ADH + EtOH</th>
<th>ADH + EtOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>33.08 (\pm) 2.90</td>
<td>28.48 (\pm) 2.45</td>
<td>33.03 (\pm) 1.08</td>
<td>27.31 (\pm) 4.68</td>
</tr>
<tr>
<td>Heart weight (g)</td>
<td>0.193 (\pm) 0.019</td>
<td>0.203 (\pm) 0.011</td>
<td>0.230 (\pm) 0.008*</td>
<td>0.293 (\pm) 0.023*</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>1.08 (\pm) 0.11</td>
<td>0.97 (\pm) 0.08</td>
<td>1.52 (\pm) 0.08*</td>
<td>1.13 (\pm) 0.20</td>
</tr>
<tr>
<td>Kidney weight (g)</td>
<td>0.325 (\pm) 0.013</td>
<td>0.270 (\pm) 0.004*</td>
<td>0.403 (\pm) 0.029*</td>
<td>0.360 (\pm) 0.058</td>
</tr>
<tr>
<td>Blood alcohol (mg/dl)</td>
<td>u.d.</td>
<td>u.d.</td>
<td>85.2 (\pm) 13.3*</td>
<td>99.9 (\pm) 24.8*</td>
</tr>
<tr>
<td>Cardiac acetaldehyde (nmol/mg)</td>
<td>9.7 (\pm) 3.4</td>
<td>7.0 (\pm) 4.1</td>
<td>60.8 (\pm) 4.9*</td>
<td>214.1 (\pm) 19.3*</td>
</tr>
<tr>
<td>Blood acetaldehyde ((\mu)M)</td>
<td>37 (\pm) 18</td>
<td>40 (\pm) 23</td>
<td>164 (\pm) 21*</td>
<td>163 (\pm) 34*</td>
</tr>
</tbody>
</table>

Mean \(\pm\) SEM, \(n = 5\) mice per group. u.d., undetectable (<2.5 mg/dl).
* \(p < 0.05\) vs. FVB group; # \(p < 0.05\) vs. FVB mice consuming ethanol.

Fig. 1. Effect of chronic ethanol administration on resting cell length and peak cell shortening in ventricular myocytes from FVB and ADH mice. (A) Representative traces depicting the effect of chronic ethanol ingestion on cell shortening from FVB myocytes. (B) Representative traces depicting the effect of chronic ethanol ingestion on cell shortening from ADH myocytes. (C) Resting cell length. (D) Peak cell shortening, indicative of peak ventricular contractility. Mean \(\pm\) SEM, \(n = 140\) cells per group. * \(p < 0.05\) versus FVB group; # \(p < 0.05\) versus FVB mice consuming ethanol.
RESULTS

General Features of FVB and ADH Mice After Chronic Intake of Ethanol or Control Diet

As shown in Table 1, ADH transgene itself did not elicit any notable effect on body, heart, and liver weights, although kidney was smaller in ADH mice. Chronic ethanol ingestion induced comparable elevation in blood levels of ethanol and acetaldehyde without affecting the body weight gain in either FVB or ADH mice. The blood ethanol levels were undetectable (<2.5 mg/dl), and blood/cardiac acetaldehyde levels were minimal in blood or hearts from the non-ethanol-ingesting animals. Chronic ethanol ingestion also resulted in cardiac hypertrophy in both mice groups, with ADH mice displaying greater severity compared with the FVB group. Ethanol ingestion elicited hepatomegaly and renal hypertrophy in FVB but not ADH mice. Although the blood acetaldehyde levels were comparably elevated in both FVB and ADH groups consuming ethanol compared with non-ethanol-consuming groups, the cardiac acetaldehyde levels were nearly 3-fold higher in ADH hearts than the FVB hearts after ethanol ingestion, validating the transgenic model of enhanced cardiac ADH activity, which is consistent with our early observations (Duan et al., 2002, 2003; Liang et al., 1999).

Effect of Chronic Ethanol Feeding on Ventricular Myocyte Mechanics

The average diastolic resting cell length (CL) was similar in myocytes from the non-ethanol-consuming FVB and ADH mice. Chronic ethanol ingestion did not affect the resting CL in myocytes from FVB group but significantly increased the resting CL in myocytes from ADH mice, an indicator of cardiac hypertrophy. The PS amplitude normalized to CL was essentially the same between the non-ethanol-consuming FVB and ADH mice. However, PS was significantly diminished in myocytes from both FVB and ADH groups after chronic ethanol ingestion, with ADH group being more sensitive to chronic ethanol-induced cardiac depression (Fig. 1). The maximal velocities of shortening/relengthening, TPS, and TR90 were comparable between the non-ethanol-consuming FVB and ADH mice. Chronic ethanol ingestion significantly depressed maximal velocity of shortening/relengthening to the same extent in both FVB and ADH mice, whereas it shortened TPS only in ADH mice and exhibited no effect on TR90 in either group (Fig. 2).

Effects of Chronic Ethanol Ingestion on Intracellular Ca\(^{2+}\) Properties and SR Ca\(^{2+}\) Load

To determine whether the disparate cardiac mechanical dysfunctions after chronic ethanol ingestion between FVB and ADH mice were related to changes in intracellular Ca\(^{2+}\) homeostasis, we used the fluorescent dye fura-2 to estimate [Ca\(^{2+}\)], and SR Ca\(^{2+}\) load in the myocytes from both groups. The time course of the fluorescence signal decay was calculated to assess intracellular Ca\(^{2+}\) decay or clearing rate. Caffeine (10 mM) was used to trigger intracellular Ca\(^{2+}\) release from SR. ADH transgene itself did not affect the intracellular Ca\(^{2+}\) decay rate, electrically stimulated increase of intracellular Ca\(^{2+}\) transients, or SR Ca\(^{2+}\) load, although it did lower the resting intracellular Ca\(^{2+}\) level in ventricular myocytes. Consistent with its effect on PS, chronic ethanol ingestion depressed electrically stimulated increase of intracellular Ca\(^{2+}\) transients and SR Ca\(^{2+}\) load without affecting resting intracellular Ca\(^{2+}\) levels and intracellular Ca\(^{2+}\) decay in either the FVB or ADH group. Furthermore, the ethanol-induced reduction in electrically stimulated increase of intracellular Ca\(^{2+}\) transients and SR Ca\(^{2+}\) load was exacerbated by the ADH transgene (Fig. 3), suggesting that the deteriorated mechanical function in myocytes from ADH mice after ethanol ingestion likely may be due, at least in part, to reduced intracellular Ca\(^{2+}\) recruiting capability in ADH myocytes in response to ethanol exposure.

Effect of Stimulation Frequency on Myocyte Shortening Between FVB and ADH Myocytes

Mouse hearts normally contract at very high frequencies (>300 beats/min at 37°C), whereas our stimulus was at 0.5 Hz (30 beats/min). To investigate possible derangement of

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**Fig. 2.** Effect of chronic ethanol administration on maximal velocity and duration of shortening and relengthening in ventricular myocytes from FVB and ADH mice. (A) Maximal velocity of shortening (+dL/dt). (B) Maximal velocity of relengthening (-dL/dt), indicative of maximal velocities of ventricular pressure rise/fall. (C) Duration of shortening (TPS), indicative of duration of systole. (D) Duration of relengthening (TR90), indicative of duration of diastole. Mean ± SEM, n = 140 cells per group. *p < 0.05 versus FVB group; #p < 0.05 versus FVB mice consuming ethanol.
cardiac E-C coupling at higher frequencies, we incrementally increased the stimulating frequency to 5 Hz (300 beat/min) and recorded the steady-state peak shortening. Cells initially were stimulated to contract at 0.5 Hz for 5 min to ensure the steady state before commencing the frequency study. All of the recordings were normalized to the PS obtained at 0.1 Hz of the same myocyte. Figure 4A shows a negative staircase in PS with increasing stimulating frequency in myocytes from non-ethanol-consuming FVB mice. However, myocytes from non-ethanol-consuming ADH mice displayed a significantly greater reduction in PS with a moderate increase in stimulus frequency (0.5 Hz and

Fig. 3. Effect of chronic ethanol administration on intracellular Ca\(^{2+}\) transients and SR Ca\(^{2+}\) load in ventricular myocytes from FVB and ADH mice. (A) Representative fura-2 traces depicting the effect of chronic ethanol ingestion on intracellular Ca\(^{2+}\) transients from FVB myocytes. (B) Representative fura-2 traces depicting the effect of chronic ethanol ingestion on intracellular Ca\(^{2+}\) transients from ADH myocytes. (C) Resting intracellular Ca\(^{2+}\) levels. (D) Intracellular Ca\(^{2+}\) decay rate. (E) Electrically stimulated rise of intracellular Ca\(^{2+}\) transient intensity from resting level. (F) Caffeine-induced SR Ca\(^{2+}\) release in myocytes isolated from FVB and ADH mice maintained on either ethanol liquid or control diet. Mean ± SEM, \(n = 61–68\) cells per group. *\(p < 0.05\) versus FVB group; #\(p < 0.05\) versus FVB mice consuming ethanol.
1 Hz) but not at higher frequencies, suggesting that intracellular Ca\(^{2+}\) resequestration may be compromised by the ADH transgene under moderate stress. Not surprisingly, chronic ethanol ingestion significantly increased the negative staircase (decrease in PS) in response to increased stimulus frequency in myocytes from both FVB and ADH mice (Fig. 4A). Unlike its effect on PS, intracellular Ca\(^{2+}\) transients, or SR Ca\(^{2+}\) load, chronic ethanol ingestion induced a comparable pattern of stimulus frequency-PS relationship in myocytes from both FVB and ADH groups, indicating that the rate of intracellular Ca\(^{2+}\) resequestration was equally affected by ethanol in both mouse groups. Last, changes in the stimulus frequency from 0.1 Hz to 5 Hz did not significantly affect diastolic resting cell length (Fig. 4B) or TPS and TR\(_{90}\) in myocytes from FVB or ADH mouse hearts (data not shown).

**DISCUSSION**

The rationale of our study is based on the hypothesis that acetaldehyde may be the ultimate toxin for alcoholic cardiomyopathy after chronic ethanol ingestion. Our major finding revealed that the cardiac-specific overexpression of

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**Effect of Chronic Ethanol Intake on Lipid Peroxidation and Protein Carbonyl Formation**

Chronic ethanol intake often is associated with enhanced oxidative stress leading to irreversible damage in membrane lipids or proteins (Preedy et al., 1999; Ren et al., 2003). Results shown in Fig. 5 indicate that the lipid peroxidation end product MDA and protein carbonyl formation were both significantly elevated in livers and hearts of the ethanol-fed mice. Liver was included because it is the major site for ethanol metabolism and conversion to acetaldehyde. The hepatic contents of MDA and protein carbonyl were similar between FVB and ADH mice consuming ethanol. However, the cardiac contents of MDA and protein carbonyl were both significantly higher in the ADH ethanol-consuming group than the FVB ethanol-consuming group, indicating elevated damage of lipid and protein in cardiac but not hepatic tissues.

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**ACETALDEHYDE AND HEART FUNCTION**
ADH leads to enhanced cardiac acetaldehyde exposure, exacerbated mechanical dysfunction, and intracellular Ca\textsuperscript{2+} dysregulation in ventricular myocytes associated with augmented damage of cardiac lipid and protein after chronic ethanol ingestion. These results have convincingly validated the role of acetaldehyde as a critical player in chronic ethanol ingestion-induced cardiac contractile defects, possibly leading to alcoholic cardiomyopathy.

The hallmark of alcoholic cardiomyopathy is cardiomegaly and compromised myocardial contractility (Patel et al., 1997; Ren and Brown, 2000; Richardson et al., 1998). This is supported by our observation of enlarged hearts and reduced myocyte shortening in both FVB and ADH mice consuming ethanol. Our data further revealed that the impaired cardiac mechanical function after chronic ethanol ingestion may be underscored by a reduction in intracellular Ca\textsuperscript{2+} release in response to electrical stimuli or caffeine (SR Ca\textsuperscript{2+} load). This is consistent with the current speculations that impaired intracellular Ca\textsuperscript{2+} homeostasis such as reduced SR Ca\textsuperscript{2+} uptake/binding and Ca\textsuperscript{2+} regulatory proteins may contribute, in large part, to altered cardiac contraction after chronic ethanol exposure (Danziger et al., 1991; Guarnieri and Lakatta, 1990; Thomas et al., 1994). More important, our study provided evidence for the first time that the severity of myocyte mechanical dysfunction and intracellular Ca\textsuperscript{2+} dysregulation after chronic ethanol intake is positively correlated to the levels of acetaldehyde in the hearts of FVB and ADH mice. It should be pointed out that the ethanol-induced myocardial contractile dysfunction also may be due to alterations of myofilament Ca\textsuperscript{2+} sensitivity rather than intracellular Ca\textsuperscript{2+} handling (Figueroedo et al., 1998). Although our earlier study indicated that acute application of acetaldehyde in vitro diminishes myocyte shortening without an appreciable inhibition on the intracellular Ca\textsuperscript{2+} transient (Ren et al., 1997), whether enhanced acetaldehyde exposure in the heart affects the myofilament Ca\textsuperscript{2+} sensitivity is not clear and deserves further study. It is somewhat surprising that the deteriorated contractile function (PS) in ADH mice after chronic ethanol ingestion was not exacerbated by enhancing the stimulus frequency. Myocytes from both FVB and ADH animals consuming ethanol displayed a similar pattern of depression in PS in response to increasing stimulus frequency, suggesting that the rate of intracellular Ca\textsuperscript{2+} resequestration (likelihood into the SR) was equally affected by ethanol in both mouse groups. This observation, in conjunction with the reduced caffeine-induced SR Ca\textsuperscript{2+} release in ADH mice after ethanol ingestion, favors the notion that SR Ca\textsuperscript{2+} load capacity rather than rate of SR Ca\textsuperscript{2+} resequestration may be responsible for exacerbated cardiac depression in ADH mice after ethanol ingestion. The fact that diastolic cell length was not significantly affected by the increasing stimulus frequency in all mouse groups tested should rule out any possible contribution of diastolic cell length to the stimulus frequency-PS response in our study. The non-ethanol-consuming ADH myocytes displayed a greater depression of PS at lower stimulus frequencies and lower resting intracellular Ca\textsuperscript{2+} levels compared with non-ethanol-consuming FVB group, which indicated that the ADH transgene itself may have an effect on intracellular Ca\textsuperscript{2+} homeostasis, however, not baseline mechanical myocyte function.

To date, the precise mechanisms of alcohol- or acetaldehyde-induced tissue and cell injury in the heart are not clear. Several hypotheses have been postulated for tissue/cell injury including oxidative damage and lipid peroxidation from alcohol and acetaldehyde oxidation and altered membrane property due to hydrophobic ethanol interaction with membrane phospholipids or protein components (Bailey et al., 1999; Cederbaum et al., 2001; Zima et al., 2001). Acute and chronic ethanol ingestion has been shown to lead to production of reactive oxygen species, oxidative stress, and enhanced peroxidation of lipids, protein, and DNA in a wide variety of organs, tissues, and cells (Bailey et al., 1999; Cederbaum et al., 2001; McDonough, 1999; Zima et al., 2001). Acetaldehyde was shown to directly enhance free radical generation by its oxidation via aldehyde oxidase and/or xanthine oxidase with concurrent accumulation of superoxide anion (Guerrero et al., 1994; Lieber, 1999; Oei et al., 1986; Zima et al., 2001). It has been reported that inhibition of xanthine oxidase or aldehyde oxidase may significantly reduce the acetaldehyde-induced lipid peroxidation and tissue damage (Shaw and Jayatilleke, 1990). Because of the link between acetaldehyde and free radical generation, damage by free radicals was examined in chronically ethanol-exposed mice. The cardiac specific ADH transgene produced even greater levels of lipid peroxidation and protein carbonyls in the hearts but not livers of alcohol-treated transgenic mice. Because these are two characteristic markers of free radical damage, these results indicate that free radical formation may play an important role in alcohol- and acetaldehyde-induced cardiac damage. Another enzyme that may contribute to acetaldehyde metabolism and free radical production is the ethanol-inducible isoform of cytochrome P-450, namely CYP2E1. Recent evidence from our lab indicated that the acetaldehyde-induced cardiac toxicity and contractile dysfunction in ventricular myocytes may be prevented by inhibition of CYP2E1 with diallyl sulfide (Aberle and Ren, 2003). Although it is not certain to what extent CYP2E1 may contribute to enhanced free radical production after ethanol ingestion, the metabolism of acetaldehyde through these enzymatic pathways may play an important role in the production of oxidative stress leading to cardiac pathology in alcoholism. In addition to the potential interactions with xanthine oxidase, aldehyde oxidase, and CYP2E1, acetaldehyde is capable of stimulating peroxidative reactions by depleting cellular reduced glutathione (Lieber, 1988). It has been shown that the ADH inhibitor pyrazole prevents the decrease in reduced glutathione levels that results from ethanol intake, whereas inhibition of aldehyde dehydrogenase by disulfiram potentiates the effect (Vina et al., 1980).
Formation of an adduct between acetaldehyde and the glutathione precursor, L-cysteine, has been speculated to contribute to glutathione depletion (Burgunder et al., 1988). In addition to the enhanced oxidative stress and injury to membrane lipid and proteins, acetaldehyde also has been shown to impair the cardiac E-C coupling through formation of acetaldehyde-protein adducts, disruption of intracellular Ca\(^{2+}\) mobilization and membrane ion channels, and inhibition of ventricular protein synthesis (Brown et al., 1999; Harcombe et al., 1995; Morales et al., 1997; Siddiq et al., 1993). The formation of protein adducts may contribute to the pathogenesis of cardiomyopathy by provoking an immunologically based reaction or by inactivating functional proteins (e.g., those involved in cardiac E-C coupling) or by causing the adducted proteins to be selectively targeted for more rapid degradation.

In summary, the present study provides convincing evidence that elevated cardiac acetaldehyde exposure exacerbates chronic ethanol ingestion-induced cardiac mechanical and intracellular Ca\(^{2+}\) dysregulation in cardiac myocytes associated with enhanced cardiac lipid peroxidation and protein carbonyl formation, supporting a role of acetaldehyde in the onset of alcoholic cardiomyopathy. Future work assessing the link between acetaldehyde and enzymatic pathways involving xanthine oxidase, aldehyde oxidase, and CYP2E1 in cardiac free radical generation is warranted to elucidate the nature of acetaldehyde in the development of alcoholic cardiomyopathy.