CARDIAC OVEREXPRESSION OF METALLOTHIONEIN RESCUES CHRONIC ALCOHOL INTAKE-INDUCED CARDIOMYOCYTE DYSFUNCTION: ROLE OF AKT, MAMMALIAN TARGET OF RAPAMYCIN AND RIBOSOMAL P70S6 KINASE

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Abstract — Aims: Reduced insulin sensitivity following alcohol intake plays a role in alcohol-induced organ damage although its precise mechanism is undefined. This study was designed to examine the effect of cardiac overexpression of the antioxidant metallothionein on alcohol-induced cardiac contractile dysfunction and post-receptor insulin signaling. Methods: FVB and metallothionein mice were fed a 4% alcohol diet for 16 weeks. Cardiomyocyte contractile function was evaluated including peak shortening (PS), time-to-PS (TPS), and time-to-relengthening (TR90). Post-insulin receptor signaling molecules Akt, mammalian target of rapamycin (mTOR), and ribosomal p70s6 kinase (p70s6k) were evaluated using western blot analysis. Akt1 kinase activity was assayed with a phosphotransferase kit. Results: Alcohol intake dampened whole body glucose tolerance, depressed PS, shortened TPS, and prolonged TR90, which were abrogated by metallothionein with the exception of glucose intolerance. Our results revealed reduced expression of total Akt, phosphorylated mTOR, and phosphorylated p70s6k-to-p70s6k ratio as well as Akt1 kinase activity in alcohol consuming FVB mice. Phosphorylated Akt, total mTOR, and phosphorylated p70s6k were unaffected by alcohol. Metallothionein ablated reduced Akt protein and kinase activity with input from insulin and insulin-like growth factors under insulin-like growth factor-1 receptor (IGF1R). Conclusion: In summary, our data suggest that chronic alcohol intake interrupted cardiac contractile function and Akt/mTOR/p70s6k signaling. Akt but unlikely mTOR and p70s6k may contribute to metallothionein-elicited cardiac protective response.

INTRODUCTION

Chronic alcoholism is a common medical, economical, and social problem. Nearly 50% of alcoholics develops a unique form of dilated cardiomyopathy, namely alcoholic cardiomyopathy manifested by ventricular dysfunction, heart failure, and ultimately cardiac death (Fernandez-Sola et al., 1994; Zhang et al., 2004). Chronic alcoholism-induced cardiac morphological and functional damage, if sustained, often contribute to the end-points of alcoholic cardiac toxicity in a manner similar to several other toxins such as doxorubicin, cocaine, monocrotaline, and azide (Zhang et al., 2004). Several hypotheses have been postulated for alcohol-induced cardiac damage including toxicity of alcohol or its metabolite acetaldehyde, accumulation of reactive oxygen species, and fatty acid ethyl esters, modifications of lipoprotein, and apolipoprotein particles, metabolic, and exotoxic changes mediated by malabsorption, maldigestion, and secondary caloric and energy deprivation as well as potential contributions from genetic factors (Patel et al., 1997; Kucera et al., 2002; Hannuksela et al., 2002; Zhang et al., 2004). Nevertheless, none of these scenarios has been fully validated by clinical and experimental data. Recent evidence has indicated a role of improved insulin sensitivity in neurocognitive recovery and psychosocial adaptation in chronic alcoholics (Esler et al., 2001). This is in line with the observation that alcohol consumption alters insulin secretion and cardiac autonomic activity including decreased sympathetic and/or increased vagal tone (Flanagan et al., 2002). Nonetheless, the relationship between alcohol intake and insulin sensitivity has been controversial for the last few decades. Although light to moderate alcohol intake seems to reduce cardiovascular risk through increased high density lipoprotein-cholesterol (HDL-C) and insulin sensitivity, the current recommendation set forth by the American Heart Association and others to limit alcohol intake to no more than two drinks per day for men and one drink per day for women appear justified but should be cautiously promoted (Spies et al., 2001; Sesso 2001). In fact, chronic alcohol intake at levels beyond moderate drinking has been associated with development of insulin resistance syndrome (Vernay et al., 2004). It is believed that cells fail to acquire sufficient trophic input from insulin and insulin-like growth factors under insulin-resistant state, leading to oxidative cell injury en route to apoptosis (Ebadi et al., 1997; Connor and Dragunow 1998). However, changes of insulin signaling cascade under alcoholism is still rather vague.

The aim of our present study was 2-fold. First, we wished to examine the impact of chronic alcohol ingestion on cardiac contractile function and post-insulin receptor signaling mechanism including Akt, mammalian target of rapamycin (mTOR), and ribosomal p70s6 kinase in cardiomyocytes. Since enhanced oxidative stress and reduced antioxidant defense following alcohol ingestion have been shown to contribute to cardiac contractile defect (Preedy et al., 1999; Spies et al., 2001; Hintz et al., 2003; Zhang et al., 2003), we tested the role of cardiac overexpression of the heavy metal scavenger metallothionein on cardiomyocyte contractile function and insulin signaling under alcohol influence.

MATERIALS AND METHODS

Experimental animals and chronic alcohol administration

The animal procedures used in this study were approved by our institutional Animal Use and Care Committee. Transgenic mice with a 10-fold cardiac-specific overexpression of...
metallothionein driven by mouse α-MHC promoter were employed as previously described (Kang et al., 1997; Fang et al., 2005). Metallothionein has been known as a powerful thio-rich scavenger of reactive oxygen species (ROS) especially hydroxyl radicals (Kang 1999). Animals were housed in individual cages under temperature- and circadian (12-h light/dark) control with free access to tap water. In this study, 3-month-old adult male FVB and metallothionein mice were introduced to a nutritionally complete liquid diet (Shake and Pour Bioserv Inc., Frenchtown, NJ, USA) for a 1-week acclimation period. The use of a liquid diet is largely based on the notion that ethanol self-administration did not induce nutritional deficiency and stress compared with forced-feeding, intravenous injection, and aerosolized inhalation (Keane and Leonard 1989). Upon completion of the acclimation period, half of the FVB and metallothionein mice were maintained on the regular liquid diet (without ethanol), and the remaining half began a 16-week period of isocaloric 4% (v/v) ethanol diet pair-feeding regimen.

**Intraperitoneal glucose tolerance test (IPGTT)**

Following 16 weeks of ethanol or control diet feeding, mice were fasted for 12 h before an intraperitoneal injection of glucose (2 g/kg body weight). Blood glucose levels were determined by clipping the mouse tail immediately before glucose challenge, as well as at 15, 30, 60, and 120 min thereafter. Blood glucose levels were determined using an ACCU-CHEK Advantage Glucose Analyzer (Roche Diagnostics Corporation, IN, USA) (Fang et al., 2005).

**Cell isolation**

After ketamine/xylazine sedation, hearts were rapidly removed from anesthetized mice and mounted onto a temperature-controlled (37°C) Langendorff system. After perfusing with a modified Tyrode solution (Ca2+ free) for 2 min, the heart was digested for 16–20 min with 0.9 mg/ml Liberase Blendzyme 4 (Hoffmann-La Roche Inc., Indianapolis, IN, USA) in the modified Tyrode solution. The modified Tyrode solution (pH 7.4) contained the following (in mM): NaCl 135, KCl 4.0, MgCl2 1.0, HEPES 10, Na2HPO4 0.33, glucose 10, butanedione monoxime 10, and the solution was gassed with 5% CO2—95% O2. The digested heart was then removed from the cannula and left ventricle was cut into small pieces in the modified Tyrode solution. Tissue pieces were gently agitated and pellet of cells was resuspended. Extracellular Ca2+ was added incrementally back to 1.20 mM over a period of 30 min. Isolated myocytes were used for experiments within 8 hours of isolation. Only rod-shaped myocytes with clear edges were selected for mechanical and intracellular Ca2+ studies (Hintz et al., 2003).

**Cell mechanics**

Mechanical properties of myocytes were assessed using an IonOptix® soft-edge MyoCam system (IonOptix, Milton, MA, USA) as described previously (Hintz et al., 2003). Myocytes were placed in a chamber mounted on the stage of an Olympus IX-70 microscope and superfused (~2 ml/min at 25°C) with a buffer containing (in mM): 131 NaCl, 4 KCl, 1 CaCl2, 1 MgCl2, 10 glucose, and 10 HEPES. Myocytes were field stimulated at 0.5 Hz unless otherwise stated. Cell shortening and relengthening were assessed using the following indices: peak shortening (PS), time-to-PS (TPS), time-to-90% relengthening (TR90), and maximal velocities of shortening/relengthening (±dl/dt).

**Western blot analysis**

Membrane proteins from cardiomyocytes were extracted as described (Fang et al., 2005). In brief, myocytes were lysed in a lysis buffer containing 20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 0.1% SDS, 20 mM NaF, 2 mM Na3VO4, and 1% protease inhibitor cocktail. Samples were then sonicated for 15 sec and centrifuged at 4500×g for 20 min at 4°C. The protein concentration of the supernatant was evaluated using the Protein Assay Reagent (Bio-Rad, Hercules, CA, USA). Equal amount (50 μg protein/lane) protein and prestained molecular weight marker (Gibco-BRL, Gaithersburg, MD, USA) were loaded onto 10 or 7% SDS-polyacrylamide gels in a minigel apparatus (Mini-PROTEAN II, Bio-Rad), separated and transferred to nitrocellulose membranes (0.2 μm pore size). Membranes were incubated for 1h in a blocking solution containing 5% non-fat milk in Tris-buffered saline (TBS) before being washed in TBS-Tween (TBS-T) and incubated overnight at 4°C with anti-Akt1 (1:1000), anti-phospho-Akt (pAkt, Thr308, 1:1000), anti-mTOR (1:1000), anti-phospho-mTOR (pmTOR, Ser2448, 1:1000), anti-p70s6k (1:1000), and anti-phospho-p70s6k (pp70s6k, Thr389, 1:1000) antibodies. All antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Following incubation with the primary antibodies, blots were incubated with either anti-mouse or anti-rabbit IgG HRP-linked antibodies at a dilution of 1:5000 for 60 min at room temperature. Immunoreactive bands were detected using the Super Signal West Dura Extended Duration Substrate (Pierce, Milwaukee, WI, USA). The intensity of bands was measured with a scanning densitometer (model GS-800; Bio-Rad) coupled with Bio-Rad PC analysis software (Ren et al., 2003). For all western blot analyses, β-actin (1:5000) was used as the loading control.

**Akt1 immunoprecipitation and kinase assay**

Akt1 kinase activity was measured using a phosphotransferase enzyme activity kit obtained from Upstate Biotechnology (Lake Placid, NY, USA). The samples were prepared in a manner similar to that used for western blot. The anti-Akt1 antibody (4 μg) was incubated with 25 μl of protein G-Agarose in a reaction buffer containing 50 mM Tris–HCl, 1 mM EDTA, 1 mM EGTA, 0.5 mM Na3VO4, 0.1% (v/v) 2-mercaptoethanol, 1% Triton X-100, 50 mM NaF, 5 mM sodium pyrophosphate, 0.1 mM PMSF, and 1% protease inhibitor cocktail (pH 7.5) at 4°C overnight. Pellets were rinsed three times with the reaction buffer. The Akt1 antibody-protein G-Agarose complex was incubated with 300 μg heart tissue lysate at 4°C at 90 min. The complex was then washed three times with the reaction buffer, twice with a washing buffer containing 50 mM Tris–HCl, 1mM EGTA, 0.03% (w/v) Brij-35, 0.1% (v/v) 2-mercaptoethanol, pH 7.5, and twice with an assay dilution buffer provided by Upstate. The complex was incubated with an Akt/SGK
substrate peptide, a protein kinase A inhibitor peptide, and 1 μCi [γ-32P]ATP (PerkinElmer Life and Analytical Sciences, Chicago, IL, USA) in the assay dilution buffer at 30°C for 10 min. After 20 μl of 40% trichloroacetic acid was added to the supernatants, the mixture was incubated for another 5 min at room temperature before being transferred to phosphocellulose paper. The phosphocellulose paper was washed three times in 0.75% phosphoric acid, once in acetone and was placed in a scintillation vial for scintillation counting (Whitlock et al., 2000).

**Data analysis**

Data were presented as Mean ± SEM. Statistical significance (P < 0.05) for each variable was estimated by two-way analysis of variance (ANOVA) or t-test, where appropriate. A Dunnett’s test was used for post hoc analysis when required.

**RESULTS**

**General features of FVB and metallothionein mice following alcohol administration**

Chronic alcohol ingestion and metallothionein transgene did not elicit any notable effects on body, heart, liver or kidney weights as well as organ size (normalized to body weight) (Table 1). The body weight gain was comparable in FVB and metallothionein mice with or without alcohol consumption during the course of 16-week feeding period (Fig. 1A). Following acute intraperitoneal glucose challenge (2 g/kg body weight), the plasma glucose levels in non-alcohol consuming FVB or metallothionein mice started to decline after peaking at 15 min. The plasma blood glucose levels returned to near baseline value after 120 min in non-alcohol consuming mice. In alcohol consuming FVB and metallothionein mice, however, the post-challenge plasma glucose levels continued to rise and peaked at 30 min. The plasma glucose levels returned to near baseline value after 120 min in non-alcohol consuming mice. In alcohol consuming FVB and metallothionein mice, however, the post-challenge plasma glucose levels continued to rise and peaked at 30 min. The plasma glucose levels remained at much higher levels between 15 and 120 min in alcohol-consuming FVB and metallothionein mice (Fig. 1B), indicating glucose intolerance in alcohol consuming FVB or metallothionein mice. Metallothionein transgene did not affect glucose clearance. There was no difference in basal fasting plasma glucose levels among all four groups tested, excluding potential contribution of diabetes mellitus.

**Effect of alcohol intake and metallothionein on cardiomyocyte mechanical function**

Neither chronic alcohol intake nor metallothionein transgene altered resting cell length. Cardiomyocytes from alcohol consuming FVB mice displayed depressed PS, shortened TPS, and prolonged TR90 without affecting ± dl/dt. Interestingly, cardiac-specific overexpression of metallothionein abrogated alcohol-induced changes in PS.

### Table 1. General features of wild-type (FVB) and metallothionein (MT) transgenic mice after 16 weeks of ethanol (ETOH) containing or control diet

<table>
<thead>
<tr>
<th>Parameter</th>
<th>FVB (n = 5)</th>
<th>FVB+ETOH (n = 6)</th>
<th>MT (n = 5)</th>
<th>MT+ETOH (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>29.70 ± 0.80</td>
<td>28.72 ± 1.52</td>
<td>28.78 ± 0.87</td>
<td>30.14 ± 0.96</td>
</tr>
<tr>
<td>Heart weight (mg)</td>
<td>185 ± 18</td>
<td>181 ± 18</td>
<td>195 ± 37</td>
<td>226 ± 22</td>
</tr>
<tr>
<td>Heart/body weight (mg/g)</td>
<td>6.18 ± 0.44</td>
<td>6.30 ± 0.57</td>
<td>6.70 ± 1.04</td>
<td>7.26 ± 0.54</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>1.51 ± 0.11</td>
<td>1.53 ± 0.10</td>
<td>1.34 ± 0.05</td>
<td>1.55 ± 0.05</td>
</tr>
<tr>
<td>Liver/body weight (mg/g)</td>
<td>51.23 ± 3.86</td>
<td>53.65 ± 2.49</td>
<td>47.28 ± 2.94</td>
<td>50.05 ± 0.88</td>
</tr>
<tr>
<td>Kidney weight (mg)</td>
<td>411 ± 30</td>
<td>401 ± 34</td>
<td>413 ± 19</td>
<td>442 ± 19</td>
</tr>
<tr>
<td>Kidney/body weight (mg/g)</td>
<td>13.77 ± 0.64</td>
<td>13.88 ± 0.57</td>
<td>14.42 ± 0.32</td>
<td>14.27 ± 0.35</td>
</tr>
</tbody>
</table>

Mean ± SEM.
Effect of ethanol ingestion and metallothionein on stimulus frequency-to-PS relationship

Murine hearts beat at high frequencies (~450/min), whereas our baseline stimulus was only at 0.5 Hz (30/min). To probe possible derangement of cardiac contractile function at higher frequencies, stimulating frequency was increased stepwise from 0.1 Hz to 5.0 Hz (300 beat/min) and PS was recorded at steady-state. All recordings were normalized to PS value obtained at 0.1 Hz of the same myocyte. Myocytes from FVB and metallothionein mice with or without alcohol consumption exhibited somewhat similar degree of PS depression from control value (0.1 Hz) at all stimulus frequencies with the exception that PS decrease was significantly lower in myocytes from alcohol consuming mice at 1.0 Hz. Metallothionein itself exerted little effect on frequency-associated decrease in PS (Fig. 2A). Our data did not observe any change in resting cell length in response to increasing stimulus frequencies among the four mouse groups (Fig. 2B).

Protein expression of Akt, mTOR and p70s6k as well as Akt kinase activity in cardiomyocytes

Western blot analysis displayed that chronic alcohol intake significantly downregulated total Akt without affecting pAkt expression in cardiomyocytes. Enzyme activity of Akt1, the main isoform of Akt protein in the hearts (Yang et al., 2005), was also decreased by chronic drinking in cardiomyocytes. Cardiac specific overexpression of metallothionein reconciled alcohol-induced reduction in total Akt and Akt1 kinase activity without affecting Akt phosphorylation (pAkt) in the hearts. The pAkt-to-Akt ratio was significantly enhanced in alcohol-fed FVB mouse cardiomyocytes, largely due to reduced total Akt expression in these cells. Metallothionein reverted chronic alcohol drinking-induced change in pAkt-to-Akt ratio. Metallothionein itself did not affect the total Akt or pAkt expression in cardiomyocytes (Fig. 3). Result depicted in Fig. 4 (panels A, C, and E) showed that chronic alcohol intake did not alter cardiac mTOR expression, one of key insulin signaling molecules downstream of Akt (Asnaghi et al., 2004). Interestingly, mTOR phosphorylation was significantly reduced by alcohol drinking in cardiomyocytes. Metallothionein elicited little effect on total and phosphorylated mTOR in cardiomyocytes from either control or alcohol consuming mice. However, the pmTOR-to-mTOR ratio was significantly reduced in cardiomyocytes from metallothionein mice, probably due to a non-significant increase in total mTOR expression in this mouse group. Our further data revealed that chronic alcohol intake enhanced total p70s6k expression without affecting phosphorylation of p70s6k in the hearts. This is associated with a reduced pp70s6k-to-p70s6k ratio in alcohol-fed FVB mouse cardiomyocytes. Metallothionein elicited no effect on total, phosphorylated p70s6k or pp70s6k-to-p70s6k ratio in cardiomyocytes from either control or alcohol consuming mice (panels B, D, and F in Fig. 4).

Table 2. General characteristics of cardiomyocyte shortening and relengthening in FVB and metallothionein (MT) mice following 16 weeks of ethanol (ETOH) containing or control diet

<table>
<thead>
<tr>
<th>Parameter</th>
<th>FVB (n = 182)</th>
<th>FVB+ETOH (n = 182)</th>
<th>MT (n = 182)</th>
<th>MT+ETOH (n = 182)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting CL (µm)</td>
<td>108.7 ± 1.8</td>
<td>104.7 ± 1.5</td>
<td>104.9 ± 1.6</td>
<td>112.2 ± 1.6</td>
</tr>
<tr>
<td>PS (% CL)</td>
<td>4.32 ± 0.20</td>
<td>3.80 ± 0.20*</td>
<td>4.63 ± 0.16</td>
<td>4.32 ± 0.17</td>
</tr>
<tr>
<td>Maximal velocity of shortening (µm/s)</td>
<td>95.8 ± 4.9</td>
<td>91.5 ± 5.0</td>
<td>100.7 ± 4.1</td>
<td>105.0 ± 4.4</td>
</tr>
<tr>
<td>Maximal velocity of relengthening (µm/s)</td>
<td>-80.9 ± 4.9</td>
<td>-80.7 ± 4.7</td>
<td>-80.2 ± 4.1</td>
<td>-89.9 ± 4.3</td>
</tr>
<tr>
<td>TPS (ms)</td>
<td>107 ± 2</td>
<td>96 ± 2*</td>
<td>110 ± 3</td>
<td>100 ± 3</td>
</tr>
<tr>
<td>TR90</td>
<td>193 ± 7</td>
<td>238 ± 18*</td>
<td>208 ± 10</td>
<td>184 ± 9</td>
</tr>
</tbody>
</table>

Mean ± SEM; CL: cell length; *P < 0.05 versus FVB group.
DISCUSSION

The major findings from our present study revealed that chronic alcohol intake leads to compromised glucose tolerance, cardiomyocyte contractile dysfunction and altered post-receptor insulin signaling including Akt, mTOR, and ribosomal p70s6k in cardiomyocytes. Cardiac-specific over-expression of the antioxidant metallothionein abrogated alcohol-induced contractile dysfunction, reduced Akt protein expression and Akt1 enzyme activity without affecting alcohol-induced depression of pmTOR, elevation in p70s6k as well as reduced ratios in pmTOR-to-mTOR and pp70s6k-to-p70s6k in cardiomyocytes. These results provided compelling evidence for an immediate role of alcoholism in altered insulin sensitivity and a beneficial role of antioxidants in cardiac contractile function and insulin signaling mechanism following chronic alcohol intake.

Compromised myocardial contractility is a hallmark of alcoholic cardiomyopathy (Patel et al., 1997; Richardson et al., 1998; Ren and Brown 2000). This is supported by our observation of reduced PM amplitude in alcohol consuming FVB mice. Our present study revealed shortened duration of contraction (TPS) and prolonged duration of relaxation (TR90) in alcohol-consuming FVB mouse cardiomyocytes. These data were somewhat similar to our earlier studies from rat papillary muscles (Ren and Brown 2000) and FVB cardiomyocytes following 8 weeks of alcohol intake (Hintz et al., 2003). Nevertheless, some discrepancies exist among these studies. Unlike our earlier observation where decreased ± dl/dt was observed following alcohol intake (Hintz et al., 2003), result from our current study failed to identify any significant effect in ± dl/dt in cardiomyocytes following 16 weeks of chronic alcohol intake. In addition, data from the present study indicated a lessened PS depression at 1.0 Hz stimulus frequency in alcohol consuming FVB mouse cardiomyocytes, whereas our earlier report observed exacerbated PS depression was seen at all stimulating frequencies following alcohol intake (Hintz et al., 2003). Although no precise explanation can be offered at this time, the difference in duration of alcohol feeding [16 weeks in current study versus 8 weeks in earlier work (Hintz et al., 2003)] may contribute to the discrepant response in TPS, TR90, ± dl/dt, and the frequency-PS relationship. Lessened decline in PS in response to higher stimulus frequency following chronic

Fig. 3. Protein expression of total Akt and phosphorylated Akt (pAkt) as well as Akt1 kinase activity in cardiomyocytes from FVB and metallothionein (MT) mice with or with alcohol consumption. (panel A) total Akt; (panel B) pAkt; (panel C) pAkt-to-Akt ratio; and (panel D) Akt1 kinase activity. Insets: representative immunoblots of Akt and pAkt using specific anti-Akt and anti-pAkt antibodies. Mean ± SEM. n = 3–5, *P < 0.05 versus FVB group, #P < 0.05 versus FVB-ETOH group.
alcohol intake (Fig. 2A) seems to suggest that the rate of intracellular Ca\(^{2+}\) re-sequestration may be less likely affected by alcohol administration. This may be a compensatory response of alcohol-afflicted cardiomyocytes or may simply reflect a relatively lower (by ~8\%, data not shown) baseline PS value at 0.1 Hz in alcohol afflicted myocytes. While several scenarios may be provided for alcohol-elicited cardiomyocyte contractile dysfunction including ethanol or acetaldehyde toxicity, accumulation of reactive oxygen species and intracellular Ca\(^{2+}\) mishandling (Ren and Brown 2000; Duan et al., 2002; Hintz et al., 2003; Zhang et al., 2003; Zhang et al., 2004), our study provided evidence for the first time that impaired insulin sensitivity and insulin signaling may underscore chronic alcohol intake-elicited cardiomyocyte contractile dysfunction. Data from our study revealed compromised whole body glucose tolerance and altered Akt/mTOR/p70s6k signaling in cardiomyocytes following chronic drinking. Insulin resistance itself has been known to lead to cardiomyocyte contractile dysfunction associated with impaired post-receptor insulin signaling mechanism (Fang et al., 2005). Akt is a key cell survival factor, the reduction of which may directly contribute to cardiac contractile dysfunction (Condorelli et al., 2002). mTOR is a central regulator of ribosome biogenesis, protein synthesis, and cell growth. It is believed that mTOR controls translation machinery in response to amino acids and growth factors, via activation of p70s6k and inhibition of eIF-4E binding protein (4E-BP1) (Asnaghi et al., 2004). All three molecules Akt, mTOR, and p70s6k may be activated by insulin, nutrients, and growth factors. A large array of evidence supports the role of these molecules in cell signaling related to cell function, growth and survival (Asnaghi et al., 2004; Fang et al., 2005; Rota et al., 2005). Chronic alcohol intake reduces Akt levels, Akt1 enzyme activity, reduced mTOR phosphorylation,
pp70s6k-to-p70s6k ratio, and enhanced inactive p70s6k in cardiomyocytes, suggesting disrupted insulin signaling at multiple levels following chronic drinking. It can be speculated that enhanced p70s6k protein expression may serve as compensatory mechanisms for depressed cardiac Akt expression/enzyme activity, as observed recently in our lab (Li et al., 2005). It should be pointed out that the somewhat disparate response among Akt, mTOR, and p70s6k upon alcohol intake may also indicate presence of an alternative pathway(s) for alcohol-induced insulin response. For example, protein kinase C may be activated by ethanol and acetaldehyde (Wyatt et al., 2000), which subsequently activate p70s6k (Ghosh et al., 2004). Furthermore, alcohol and acetaldehyde have been demonstrated to turn on mitogen-activated protein kinase (MAPK) pathways (Zhang et al., 2004), which may exert an indirect effect on cardiomyocyte insulin sensitivity and therefore impair cardiomyocyte contractile function. Further study is warranted to evaluate the contribution of these potential mechanisms.

Our data revealed that cardiac overexpression of metallothionein abrogates mechanical defects associated with chronic alcoholic intake. Although the exact mechanism behind this beneficial effect is not completely understood at this time, it may be speculated that metallothionein may scavenge free radicals to improve insulin sensitivity in cardiomyocytes. This notion is supported by improved Akt expression and Akt1 enzyme activity in metallothionein mouse cardiomyocytes. The lack of effect on mTOR and p70s6k in metallothionein mouse hearts may indicate either non-factors of these signaling molecules in metallothionein-elicited cardiac protection or simply ineffectiveness of the antioxidant on certain compensatory machineries in the post-receptor insulin-signaling cascade. In fact, metallothionein itself reduced pmTOR-to-mTOR ratio, in a manner similar to chronic alcohol drinking. One of the limitations in our current study is lack of measurement of reactive oxygen species, which may play a role in metallothionein-elicited improvement on cardiomyocyte function and Akt expression/enzyme activity. Our previous reports have demonstrated that alcohol directly leads to oxidative stress, apoptosis, and protein damage (Ren et al., 2002; Hintz et al., 2003). In addition, we also observed beneficial roles of metallothionein against heart damage due to enhanced oxidative stress under diabetic or aging conditions (Ye et al., 2003; Fang et al., 2006).

In summary, our present study provided convincing evidence that chronic alcohol intake is associated with impaired glucose tolerance, cardiomyocyte contractile dysfunction, and disrupted cardiac Akt/mTOR/p70s6k signaling. Our data also revealed that enhanced antioxidant defense in the hearts may rescue cardiac contractile dysfunction and compromised post-receptor insulin signaling possibly at the level of Akt. These finding suggests that antioxidants and insulin sensitizers such as vitamin and peroxisome proliferator-activated receptor-γ (PPAR-γ) agonist may be beneficial to the maintenance of ventricular function in chronic alcohols. Although it may not be practical to overexpress metallothionein clinically in alcohols, our data suggest that the artificial ‘jack-up’ of the antioxidant defense such as using metallothionein or superoxide dismutase enzyme can make up for the compromised antioxidant defense following chronic alcohol intake. Further study is warranted to understand the underlying cellular mechanisms associated with alcohol-induced loss of insulin sensitivity and the role of insulin resistance in the pathogenesis of alcoholic cardiomyopathy.

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