Ca^{2+}-Regulatory Muscle Proteins in the Alcohol-Fed Rat

Kay Ohlendieck, Shona Harmon, Michael Koll, Alistair G. Paice, and Victor R. Preedy

Alcoholic myopathy is characterized by muscle weakness and difficulties in gait and locomotion. It is one of the most prevalent skeletal muscle disorders in the Western hemisphere, affecting between 40% and 60% of all chronic alcohol misusers. However, the pathogenic mechanisms are unknown, although recent studies have suggested that membrane defects occur as a consequence of chronic alcohol exposure. It was our hypothesis that alcohol ingestion perturbs membrane-located proteins associated with intracellular signalling and contractility, in particular those relating to calcium homeostasis. To test this, we fed male Wistar rats nutritionally complete liquid diets containing ethanol as 35% of total dietary energy. Controls were pair-fed identical amounts of the same diet in which ethanol was replaced by isocaloric glucose. At the end of 6 weeks, rats were killed and skeletal muscles dissected. These were used to determine important ion-regulatory skeletal muscle proteins including sarcalumenin (SAR), sarcoplasmic-endoplasmic reticulum Ca^{2+}-adenosine triphosphatase (ATPase) (SERCA1), the junctional face protein of 90 kd (90-JFP), α_1- and α_2-dihydropyridine receptor (α_1-DHPR and α_2-DHPR), and calsequestrin (CSQ) by immunoblotting. The relative abundance of microsomal proteins was determined by immunoblotting using the enhanced chemiluminescence (ECL) technique. The data showed that alcohol-feeding significantly reduced gastrocnemius and hind limb muscle weights ($P < .05$ in both instances). Concomitant changes included increases in the relative amounts of SERCA1 ($P < .05$) and Ca^{2+}-ATPase activity ($P < .025$). However, there were no statistically significant changes in either SAR, 90-JFP, α_1-DHPR or α_2-DHPR ($P > .2$ in all instances). Reductions in CSQ were of marginal significance ($P = .0950$). We conclude that upregulation of SERCA1 protein and Ca^{2+}-ATPase activity may be an adaptive mechanism and/or a contributory process in the pathology of alcohol-induced muscle disease.

CHRONIC ALCOHOL DEPENDENTS may not only suffer from cirrhosis-induced impairment of liver function, cardiac complications, and various alcohol-based psychotic disorders, but up to two thirds have severe skeletal muscle dysfunction, ie, alcohol-induced muscle disease.[1,2] Chronic alcoholic muscle disease is characterized by defects in contractility, muscle weakness, and loss of muscle mass. However, the mechanisms for this pathology are unknown.[1,2] Nevertheless, 3 lines of evidence from our group suggest that disturbances in the membrane domain may occur as a consequence of alcohol exposure. The first pertains to adduct formation, which occurs especially within the sarcolemmal region of muscle.[3,4] Secondly, reductions in ribosomal protein in alcohol-exposed muscle occurs preferentially in the subsarcolemmal region in contrast with the more moderate changes in the intermyofibrillar ribosomes.[5] Finally, as a consequence of oxidative stress, alcohol increases the concentration of the membrane located hydroperoxides, 7α-hydroperoxycholest-5-en-3β-ol (7α-OOH) and 7β-hydroperoxycholest-5-en-3β-ol 7β-OOH.[6] Furthermore, studies from other groups have also shown disturbances in the membrane domain of muscle, such as alcohol-induced decreases in Ca^{2+} transients in cultured human myotubes.[7]

In skeletal muscle fibers, excitation-contraction coupling and muscle relaxation is regulated by changes in cystosolic Ca^{2+} levels.[8] Ca^{2+} cycling through the sarcoplasmic reticulum is mediated by the junctional ryanodine receptor Ca^{2+} release channel complex and the Ca^{2+}adenosine triphosphatase (ATPase) units.[9] Following voltage sensing by the α_1-subunit of the dihydropyridine receptor,[10] direct physical triad receptor coupling triggers Ca^{2+} efflux through the ryanodine receptor.[11] The energy-dependent reuptake of Ca^{2+} ions is achieved by Ca^{2+} pumps of the longitudinal tubules and the terminal cisternae.[12] Within the lumen of the sarcoplasmic reticulum, the high-capacity Ca^{2+}-binding protein calsequestrin (CSQ)[13] acts as an ion reservoir and endogenous regulator of Ca^{2+} efflux.[14] Other luminal Ca^{2+}-binding proteins are represented by sarcalumenin (SAR)[15] and calreticulin (CAL).[16]

Since ethanol causes biochemical abnormalities within the membrane domain of muscle,[3-7] it was our hypothesis that chronic alcohol ingestion perturbs key components of calcium homeostasis. This is a reasonable supposition, since abnormal Ca^{2+} handling is involved in various skeletal muscle disorders.[17] Such damage may contribute to potential pathways of alcohol-induced tissue damage including functional deficiencies such as weakness (for example see, Odermatt et al[18]). To determine the relative expression of key Ca^{2+}-regulatory muscle proteins, we have performed immunoblotting of microsomal proteins derived from control and chronic alcohol-exposed skeletal muscle fibers. To unequivocally identify the various muscle proteins, established antibodies were used for highly specific immuno decoration of the α_1- and α_2-subunit of the transverse-tubular DHPR, the Ca^{2+}-binding proteins CSQ of the terminal cisternae and SAR of the longitudinal tubules, as well as the Ca^{2+}-ATPase. For the internal standardization of immunoblots, the expression of the abundant dystrophin-associated surface marker β-dystroglycan was employed.[19]

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Table 1. Composition and Energy Values of Control and Alcohol-Containing Liquid Diets

	Content (g)	Total kcal	Total kJ
Alcohol diet			
Water	126	0.0	0.0
Fresubin	600	586	2,448
Added glucose	0.0	0.0	0.0
Casein	15.0	60	251
Orovite (sucrose)	2.46	9	39
Alcohol (61.8 ml)	49.08	348	1,457
Total	1,063	1,003	4,194
Control diet			
Water	126	0.0	0.0
Fresubin	600	586	2,448
Added glucose	93	349	1,458
Casein	15.0	60	251
Orovite (sucrose)	2.46	9	39
Alcohol	0	0	0
Total	1,106	1,004	4,196

NOTE. Calorific values were calculated as follows: ethanol = 7.1 kcal/g; carbohydrate = 3.8 kcal/g; protein = 4.0 kcal/g; fat = 9.0 kcal/g; and 4.18 kJ was assumed to be = 1.0 kcal.

MATERIALS AND METHODS

Materials

Protease inhibitors and acrylamide stock solutions were obtained from Boehringer-Mannheim (Lewis, UK). Chemiluminescence substrates were purchased from Pierce and Warriner (Chester, UK). Immobilon NC nitrocellulose membranes were from Millipore (Bedford, MA). Commercially available primary antibodies were obtained from Affinity Bioreagents, Golden, CO: monoclonal antibody (mAb) VIIID1$_2$ to CSQ, mAb 20A to the α_2-subunit of the DHPR, mAb XIIC4 to SAR, mAb VF1c to the 90-kd junctional face protein, mAb IIH11 to the fast SERCA1 isoform of the sarcoplasmic endoplasmic reticulum Ca^{2+}-ATPase, and pAb PA3-900 to calreticulin. Peroxidase-conjugated secondary antibodies were purchased from Chemicon (Temecula, CA). Monoclonal antibody IIID5 to the α_1-subunit of DHPR was a generous gift from Dr Kevin P. Campbell (Howard Hughes Medical Institute, University of Iowa, Iowa City, IA). All other chemicals were of analytical grade and purchased from Sigma Chemical Co (Dorset, UK).

Animal Treatments

Male Wistar rats were obtained from accredited commercial suppliers at about 60 g body weight then maintained for approximately 1 week in a Home Office–approved animal house until they weighed approximately 0.1 kg. At this stage, rats were ranked and divided into 2 groups of equal mean body weight, and designated to either controls or ethanol-treated groups. The treated rats were subjected to a Lieber-DeCarli alcohol-feeding regimen in which animals were fed a nutritionally complete liquid diet containing 35% of total dietary energy as ethanol.[20,21] Controls were pair-fed identical volumes of the same diet in which ethanol was replaced by isocaloric glucose[20,21] (Tables 1 and 2). After approximately 6 weeks, animals were killed by decapitation. Skeletal muscles were dissected and limb muscle mass determined. Dissected muscle was frozen in liquid nitrogen (−196°C) and stored at −70°C until use. For analysis, either the gastrocnemius or hind limb musculature was used. A group of rats were fed on a standard solid

Table 2. Comparison of Chow Diet and Liquid Diets Used for Chronic Alcohol Feeding

	Chow Diet (per kg)	Liquid Control (glucose) Diet (per kg)	Liquid Alcohol Diet (per kg)
Nutrients			
Energy	12.5 MJ (2,988 kcal)	3,816 kJ (912 kcal)	3,812 kJ (911 kcal)
Fat/oil	208 g	18 g	18 g
Protein	174 g	34 g	34 g
Carbohydrates	495 g	142 g	107 g
of which glucose	n/a	83 g	0
of which alcohol	n/a	0	49 g
Water	100 g	779 g	779 g
Vitamins and selected minerals			
Vitamin A	5,614 μg	243 μg	243 μg
Vitamin D	0.129 μg	136 μg	136 μg
Vitamin E	103 μg	4 μg	4 μg
Vitamin K	185 μg	26.9 μg	26.9 μg
Vitamin B_1	15.8 mg	1.16 mg	1.16 mg
Vitamin B_2	13.6 mg	1.46 mg	1.46 mg
Niacin	78.7 mg	12.9 mg	12.9 mg
Vitamin B_6	18.5 mg	1.54 mg	1.54 mg
Vitamin B_{12}	83.5 μg	1.07 μg	1.07 μg
Pantothenic acid	26.1 mg	1.88 mg	1.88 mg
Biotin	0.359 mg	53.7 mg	53.7 mg
Folic acid	4.4 mg	54 μg	54 μg
Ascorbic acid	8.0 mg	51.1 mg	51.1 mg
Calcium	8.0 g	322 mg	322 mg
Iron	110 mg	540 μg	540 μg
Iodine	0.60 mg	57.1 mg	57.1 mg

NOTE. Data to show differences between solid chow and liquid diets for selective nutrients. Chow CRM pellets diets (SDS, Witham, UK) were used. The liquid diet was made up using Fresubin (Fresenius Kabi Ltd, Mano Park, UK), glucose and casein (both Merck BDH, Lutterworth, UK), Orovite 7 (Boots Chemist), 99.9 % alcohol (Hospital Pharmacy), and water.

chow diet (ie, pelleted) ad libitum for the duration of the study for comparative purposes. These were from the same batch of rats selected for liquid feeding with either glucose or ethanol.

Liquid Diets

Fresh liquid diets used for the 6-week chronic ethanol feeding experiment were prepared on a daily basis according to the recipe described in Table 1. A food blender was used to thoroughly mix the ingredients. To prevent the possibility of ethanol precipitating the protein in the alcohol diet, absolute ethanol was the last ingredient to be added carefully, and contents were then thoroughly stirred during the addition. The composition of the diet is given in Table 2. Control and alcohol-containing diets were isolipidic, isonitrogenous, and isoenergetic (Tables 1 and 2).

The compositions of the solid chow and liquid diets are entirely different with respect to macro- and micronutrients such as vitamins, minerals, and other essential and nonessential dietary elements (Table 2). Thus, any comparisons between the rats fed ad libitum on the solid chow and animals fed on the 2 liquid diets will be potentially difficult to interpret and may be due to any one or several of over 100 dietary components (see also Table 2). For this reason, detailed and comparative biochemical analysis of the muscle from solid chow and liquid fed rats was not performed (for further details on the feeding protocols, see Preedy et al[21] and Lieber and DeCarli[22]). Rather, representative blots are presented to illustrate the fact that the liquid diet-feeding regimen had no overt effect on the gross features of immunoblotting (electrophoretic separation, migration, etc).

Subcellular Fractionation

For subcellular fractionation, muscle specimens were homogenized in 5 vol of 20 mmol/L Tris-maleate, pH 7.0, 3 mmol/L EGTA, 10% (wt/vol) sucrose using an Ultra-Turrax T25 (IKA-Labortechnik, Staufen, Germany) and subsequently crude microsomal membranes prepared by standard differential centrifugation.[19] All preparative steps were performed at 0 to 4°C in a cold room and all buffers were supplemented with a protease inhibitor cocktail consisting of 1 mmol/L EDTA, 0.2 mmol/L pefabloc, 1.4 μmol/L pepstatin A, 0.15 μmol/L aprotinin, 0.3 μmol/L E-64, 1 μmol/L leupeptin, and 0.5 μmol/L soybean trypsin inhibitor to minimize protein degradation.[23,24] Protein concentration of resuspended microsomal pellets was determined by the method of Bradford[25] using bovine serum as standards. To determine potential differences in the Ca^{2+}-ATPase activity between control and ethanol-treated microsomal preparations, a direct colourimetric assay was employed.[26]

Gel Electrophoresis

For electrophoretic separation, protein samples were mixed with an equal volume of double strength sample buffer containing 3% (wt/vol) sodium dodecyl sulfate (SDS), 8 mol/L urea, 2 mol/L thiourea Bradford, 0.05% (wt/vol) bromophenol blue, and 50 mmol/L Tris-HCl, pH 6.8, 75 mmol/L dithiothreitol (DTT),[27] and 20 μg protein run per lane using 7% (wt/vol) separation gels at a constant voltage for 280 Vh in a Mini-Protean II gel system from Bio-Rad Laboratories (Hemel Hempstead, UK).

Immunoblot Analysis

To determine alcohol-induced effects on the isoform expression level of key Ca^{2+}-regulatory proteins, muscle proteins separated by gel electrophoresis were transferred to Immobilon NC membranes according to previously described methods[28] using a Bio-Rad Mini-Protean II blotting system (Bio-Rad Laboratories). Blocking, washing, and incubation with primary and peroxidase-conjugated secondary antibodies were performed by established methods. Immunodecoration was evaluated by enhanced chemiluminescence (ECL). Densitometric scanning of ECL-blots was performed on a Molecular Dynamics 300S computing densitometer (Sunnyvale, CA) using ImageQuant V3.0 software.[24]

Statistics

All data are displayed as means ± SEM (n = 5 to 8). For differences between glucose-fed and alcohol-treated rats on liquid diets, statistical evaluation was performed by Student's *t* test for paired samples. For differences between ad libitum–fed rats on the solid pelleted chow diet, and the other 2 groups of rats on liquid diets, post-hoc statistical evaluation was performed by Student's *t* test for unpaired samples.

RESULTS

Comparison Between Chow-Fed Rats and Liquid-Fed Groups

Mean body weights were as follows with ensuing data presented as means ± SEM with *P* values pertaining to differences between ad libitum–fed rats on a solid chow diet (n = 8) versus rats fed on a nutritionally complete liquid diet containing approximately one third of total dietary energy as either glucose (n = 8) or ethanol (n = 8). Body weights (g) were as follows: ad libitum solid chow-fed, 393 ± 9; glucose-fed rats on a liquid diet, 194 ± 4 ($P < .001$); and ethanol-treated rats on a liquid diet, 184 ± 3 ($P < .001$). Gastrocnemius weights (right muscle, g) were as follows: ad libitum solid chow-fed, 1.715 ± 0.025; glucose-fed rats on a liquid diet, 1.040 ± 0.038 ($P < .001$); and ethanol-treated rats on a liquid diet, 0.781 ± 0.071 ($P < .001$). Hind limb weights (right musculature, g) were as follows: ad libitum solid chow-fed, 9.361 ± 0.274 g; glucose-fed rats on a liquid diet, 5.073 ± 0.195 ($P < .001$); and ethanol-treated rats on a liquid diet, 3.981 ± 0.159 ($P < .001$). These results reaffirms the supposition that alcohol-fed rats have muscle weights lower than those from rats fed on a solid diet ad libitum and support the notion that a pair-feeding regimen should be used to investigate the putative effects of alcohol on muscle.

Muscle from ad libitum–fed rats on the solid chow diet were not subjected to detailed biochemical analysis as comparisons with rats on liquid diet are unsound. We reiterate the entirely different nature and composition of the solid chow diet in comparisons with the liquid feeds. In simple terms, any potential difference between muscles from rats fed on the solid or liquid diets may be due to any one of a multitude of dietary components (see Table 2).

On the other hand, it is also important to show that the liquid feeding protocol did not cause overt changes in muscle protein profiles (either via overt changes in relative abundance or molecular mass, etc,) and for this reason a general comparison between representative samples from ad libitum–fed rats on the solid chow, and rats on the glucose- or ethanol-containing liquid diets are displayed on the same gel. These gels show similar patterns of expression and molecular mass of immunopositive proteins in muscles from rats fed on solid or liquid diets.

Comparison Between Glucose-Fed Control and Alcohol-Treated Rats on Liquid Diets

Chronic ethanol feeding reduced body weight and skeletal muscle weight (Fig 1). There were almost identical propor-

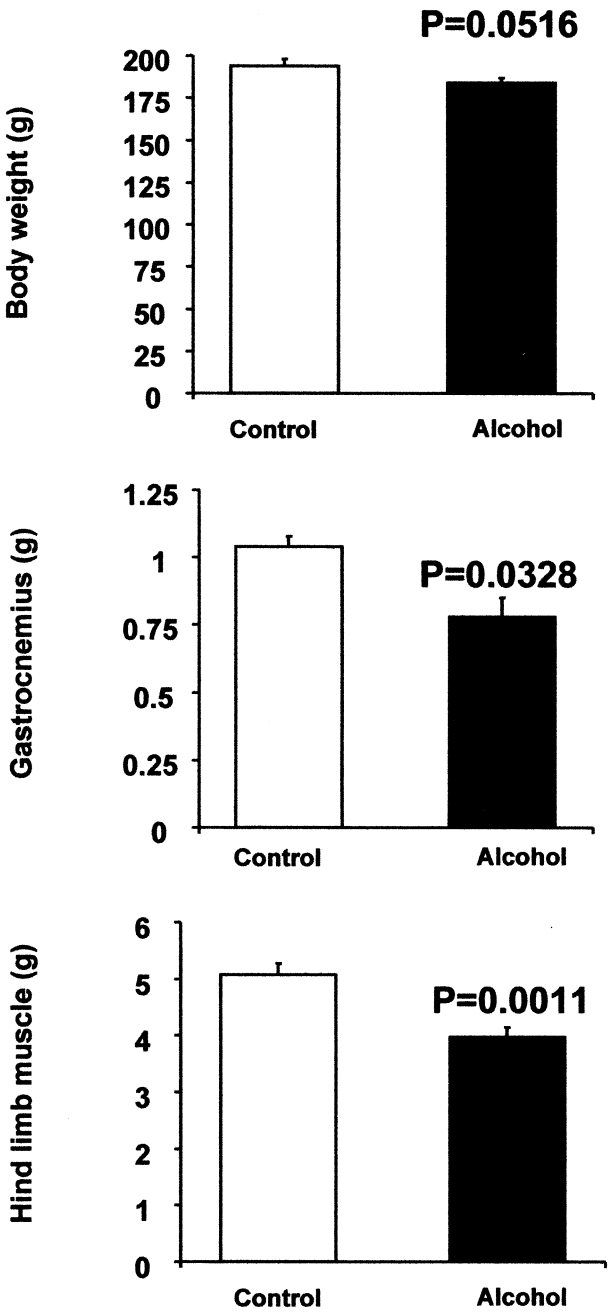

Fig 1. Body and muscle weights in skeletal muscle of ethanol-fed rats. Rats were fed a nutritionally complete liquid diet containing ethanol as 35% of total calories. Controls were pair-fed the same diet in which ethanol was replaced by isocaloric glucose. At the end of the study, rats were killed and the muscles dissected out. Histograms represent the mean ± SEM (n = 8 pairs). Notations above each histogram of the ethanol group refer to differences from the control groups. Differences between means were assessed with Student's *t* test for paired samples. For details on the changes in the muscle from rats on the solid chow diet, see Results.

tional changes in hind limb muscle and gastrocnemius consistent with previously reported changes in this model (Fig 1).

Since it is postulated that ethanol and/or its metabolites have a modulatory effect on membrane fluidity and ion-regulatory membrane proteins, we determined the expression of key microsomal components of Ca^{2+}-handling from chronic alcohol exposed skeletal muscle. As illustrated in Fig 2, the Coomassie-stained protein band pattern of microsomal preparations from ad libitum–fed and glucose-fed control and myopathic muscle samples were relatively comparable. Since individual protein bands in Coomassie-stained gels may pertain to numerous molecular species, changes in specific isoforms of membrane proteins were identified using immunoblotting. Established antibodies to the α_1- and α_2-subunit of the dihydropyridine receptor (Figs 3 and 4, respectively), CSQ (Fig 5), SAR (Fig 6), the 90-kd junctional face membrane protein (Fig 7), the sarcoplasmic reticulum Ca^{2+}-ATPase (Fig 8), and the surface membrane marker β-dystroglycan (Fig 9) were employed. In conjunction with Fig 8, the enzyme activity of the sarcoplasmic reticulum Ca^{2+} pump was also determined (Fig 10).

Immunoblot analysis using monoclonal antibodies to the 2 main subunits of the transverse-tubular voltage sensor, the principal α_1-subunit and the auxiliary α_2-subunit of the DHPR revealed no alcohol-related changes in their expression. Neither the intensity of immunolabeling nor the relative position in comparison to molecular mass standard differed between normal and alcoholic myopathy specimens (Figs 3 and 4). The major Ca^{2+}-binding protein of the luminal sarcoplasmic reticulum, CSQ, seems to be only marginally affected by alcohol-induced modifications of muscle membrane systems (Fig 5). Evaluation of immunodecoration revealed a slight decrease in this terminal cisternae component. SAR of apparent 160 kd

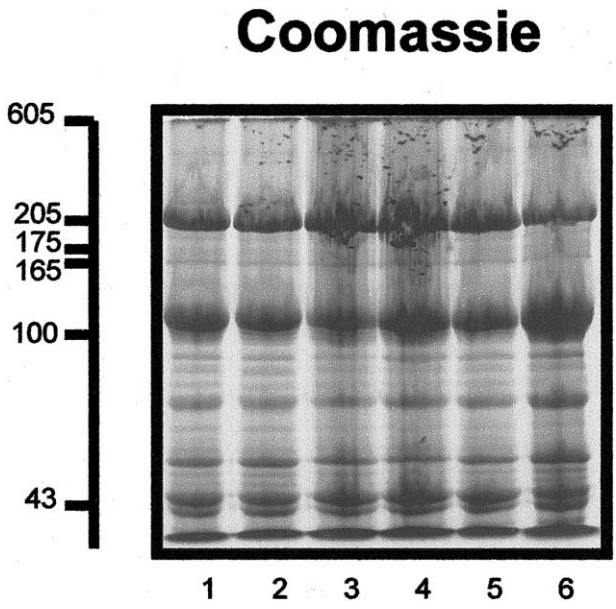

Fig 2. Coomassie-stained gel of microsomal muscle protein fraction from control and ethanol-fed rats. Shown is a Coomassie-labeled gel, whereby lanes 1 and 2, 3 and 4, and 5 and 6 are the microsomal fraction isolated from rats representing the control-fed, ethanol-fed, and ad libitum animal groups, respectively. Muscle was obtained from rats fed on a solid diet ad libitum, or rats fed on a nutritionally complete liquid diet containing either glucose or ethanol as 35% of total dietary energy.

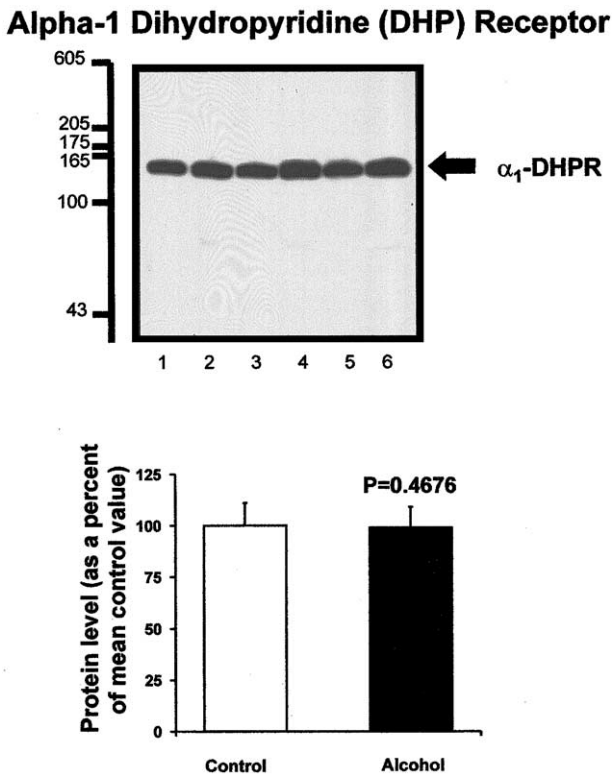

Fig 3. Immunoblot: α_1-subunit of the DHPR in muscle from control and ethanol-fed rats. Shown is an immunoblot labeled with an antibody to the α_1-DHPR, whereby lanes 1 and 2, 3 and 4, and 5 and 6 are the microsomal fraction isolated from rats representing the control-fed, ethanol-fed, and ad libitum animal groups, respectively. The relative position of the immuno-decorated bands is marked by an arrow. The position of molecular mass standards ($\times 10^{-3}$) is indicated at the left. Histograms pertain to glucose-fed control and ethanol-fed rats and are displayed as the mean ± SEM (n = 5 pairs). *P* values are displayed over the histograms. Detailed analysis of the muscle from rats on the solid chow diet was not carried out as explained in the main text (Methods and Discussion sections) and are displayed for comparative purposes only.

represents a Ca^{2+}-binding protein (and possibly luminal Ca^{2+}-transporter) predominantly localized to the longitudinal tubules of the sarcoplasmic reticulum and its expression was not significantly altered in microsomes from myopathic rats (Fig 6). Immunolabeling of the ubiquitous Ca^{2+}-binding protein CAL did not reveal distinct enough signals for proper statistical evaluation. However, our preliminary analysis indicates that the overall expression levels of this protein have not been changed in alcohol-exposed skeletal muscle microsomes (not shown). Since antibodies to the RyR1 isoform of the ryanodine receptor Ca^{2+}-release channel did not sufficiently cross-react with rat muscle for proper immuno decoration, we used the 90-kd junctional face membrane protein as a triad marker protein. This protein was previously shown to exist in a very close neighborhood relationship with the junctional Ca^{2+}-release channel complex and is therefore a suitable substitute for the RyR1 protein. The immunoblot analysis shown in Fig 7 demonstrates that chronic exposure to alcohol had no significant effect on this sarcoplasmic reticulum protein. The most important Ca^{2+}-removal system during skeletal muscle relaxation is the sarcoplasmic reticulum Ca^{2+}-ATPase and immuno decoration of the fast-twitch isoform SERCA1 with monoclonal antibody IIH11 clearly revealed an upregulation of this ion pump in myopathic specimens (Fig 8). Statistical analysis demonstrates an additional 65% increase in the relative expression level of SERCA1 ($P = .0113$). In agreement with the increased relative abundance of the SERCA1 isoform, there is an upregulation of its activity by approximately 15% ($P = .0357$; Fig 10). For internal standardization of our immunoblotting, we employed the abundant microsomal protein β-dystroglycan. As illustrated in Fig 9, normal control and myopathic membrane preparations exhibit very comparable levels of this sarcolemmal marker demonstrating equal loading in individual gel lanes. Thus, the increase in the expression of the Ca^{2+}-ATPase protein are not artefacts of gel electrophoresis procedures, protein transfers or immuno decoration methodology, but represent the effect of chronic alcohol exposure on key Ca^{2+}-regulatory membrane proteins.

DISCUSSION

Methodological Considerations

An important element of our study was the employment of a pair-feeding regimen, which facilitated the direct comparison

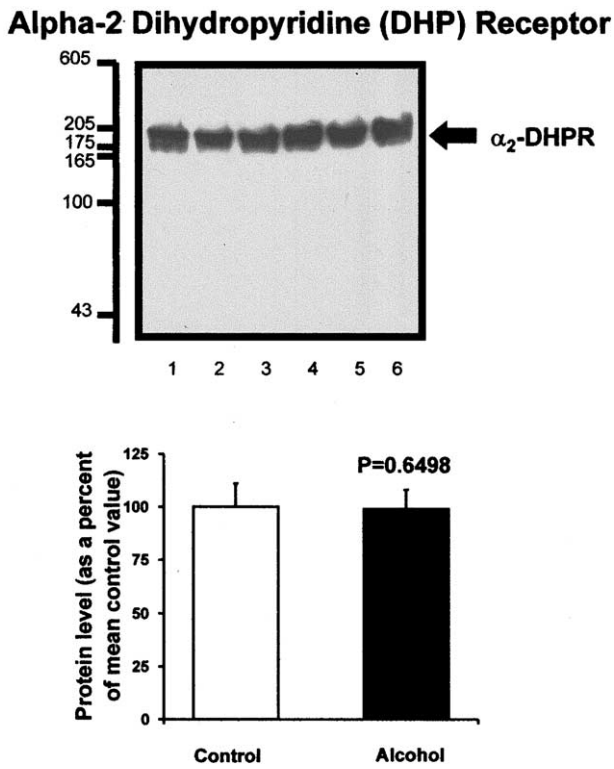

Fig 4. Immunoblot: α_2-subunit of the DHPR in muscle from control and ethanol-fed rats. Shown is an immunoblot labeled with an antibody to the α_2-DHPR, whereby lanes 1 and 2, 3 and 4, and 5 and 6 are the microsomal fraction isolated from rats representing the control-fed, ethanol-fed, and ad libitum animal groups, respectively. For other details see legend to Fig 3.

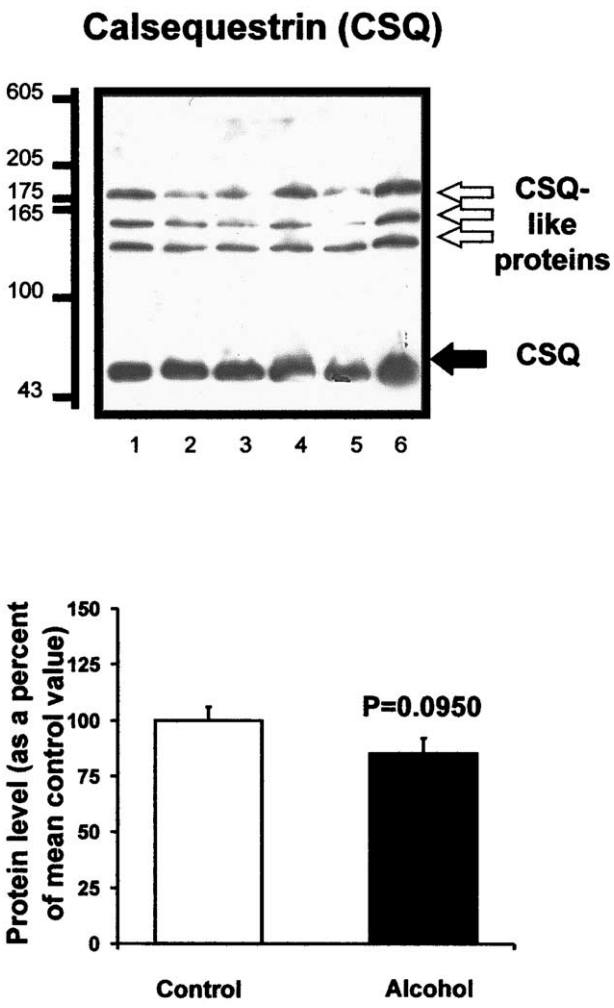

Fig 5. Immunoblot: CSQ in muscle from control and ethanol-fed rats. Shown is an immunoblot labeled with an antibody to CSQ, whereby lanes 1 and 2, 3 and 4, and 5 and 6 are the microsomal fraction isolated from rats representing the control-fed, ethanol-fed, and ad libitum animal groups, respectively. For other details see legend to Fig 3.

between glucose-fed control and ethanol-fed treated rats. The purpose of employing a pair-feeding protocol pertains to the anorexic effects of consuming large amounts of alcohol as described previously.[21,22] Rats fed alcohol-containing diets consume considerably less food than their counter parts fed diets without alcohol.[20-22] Thus, potentially, effects observed in alcohol-fed rats may be due to reduced dietary intakes rather than alcohol toxicity per se.[20-22] To resolve this, control rats were given identical amounts of the same liquid diet in which ethanol was replaced by isocaloric glucose.[21,22] To confirm whether the pair feeding was necessary we analyzed muscle from a group of rats fed a solid chow diet ad libitum for the duration of the study. At the end of the 6 weeks, muscle from these rats were heavier than those from either the glucose-fed controls or ethanol-fed rats.

We are unable to explain why a study reported by another group showed no change in skeletal muscle Ca^{2+}-ATPase activity in experimental chronic alcoholic myopathy.[29] Changes in enzyme activity in our model were small though statistically significant. However, consideration needs to be given to the fact that enzyme activities assayed in artificial conditions in vitro do not reflect the precise regulatory control mechanisms occurring in the intact cell in vivo. We are confident of an upregulation as much greater changes occurred after analysis of the SERCA1 protein using immunoblotting.

It is important to point out that changes in protein expression of SERCA1 and enzyme activity per se will not by themselves contribute to the myopathy as other processes are involved. Hitherto, we believed that disturbances in the rate of protein turnover were central steps in the etiology of the myopathy.[30-33] Subsequently, we have shown that the changes are more complex, encompassing intervening steps such as protein adduct formation, altered protease activities, and increased proto-oncogene expression, free radicals, and other pathogenic factors such as membrane damage.[1,2,34-38] Nevertheless, it is of interest to compare the magnitude of these changes in SERCA1 with

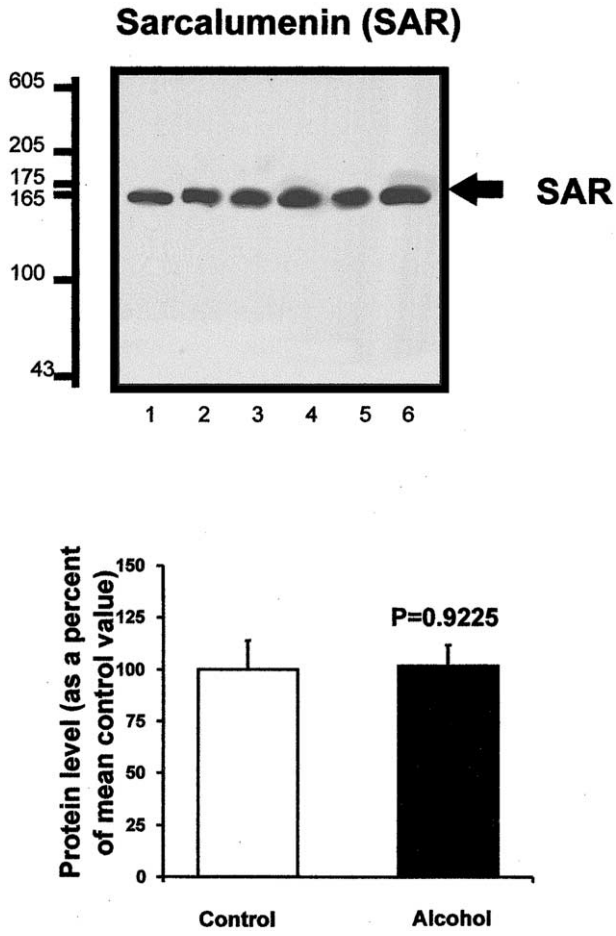

Fig 6. Immunoblot: SAR in muscle from control and ethanol-fed rats. Shown is an immunoblot labeled with an antibody to SAR, whereby lanes 1 and 2, 3 and 4, and 5 and 6 are the microsomal fraction isolated from rats representing the control-fed, ethanol-fed, and ad libitum animal groups, respectively. For other details see legend to Fig 3.

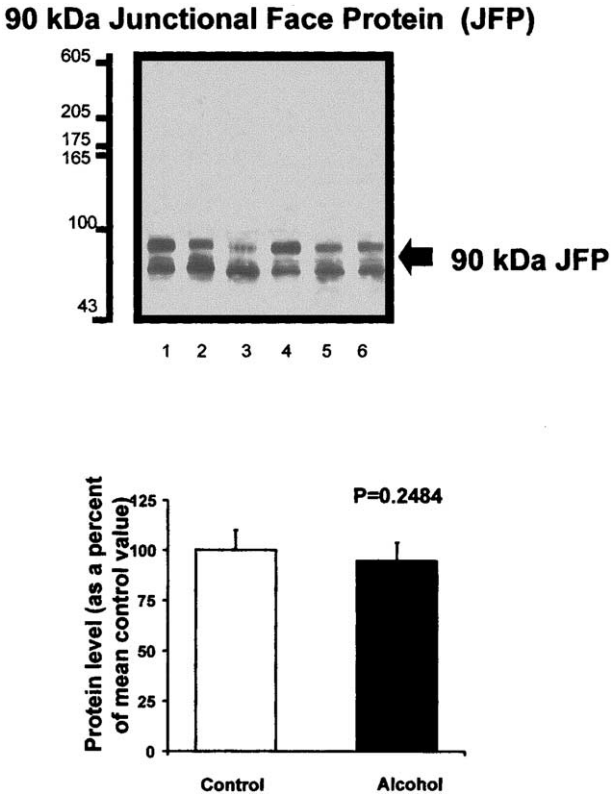

Fig 7. Immunoblot: 90-kd junctional face membrane protein in muscle from control and ethanol-fed rats. Shown is an immunoblot labeled with an antibody to JFP, whereby lanes 1 and 2, 3 and 4, and 5 and 6 are the microsomal fraction isolated from rats representing the control-fed, ethanol-fed, and ad libitum animal groups, respectively. For other details see legend to Fig 3.

other reported myopathologies (most of which show decreases in Ca^{2+}-ATPase activity or protein). Thus, in ischemia enzyme activities decrease in vitro, by about 25%.[39] In experimental heart failure, skeletal muscle SERCA protein (albeit the 2a isoform) has been reported to decrease by 16% in slow-twitch muscle at a significance level of $P < .001$.[40]

We must also view the refractory nature of the non-SERCA proteins (Figs 3 through 7 and 9) in a more positive light. Remarkably, these proteins are conserved in both abundance and immunogenicity even in the presence of high levels of circulating ethanol and acetaldehyde (for example see Worrall et al[3] and Niemala et al[4]). Previous studies have shown the presence of protein adducts, especially around the subsarcolemmal regions so a downregulation or loss of immunoreactivity of such non-SERCA proteins would be expected.

The abundance of SERCA1 is clearly increased in the alcohol-fed rats compared to the isocaloric glucose-fed rats (Fig 8). We have interpreted this observation to indicate that alcohol-treatment induced an upregulation of the sarcoplasmic reticulum Ca^{2+}-ATPase. However, it appears that the SERCA1 content in the alcohol-treated rats appears to be very similar to those rats fed ad libitum. An alternative interpretation of this data would be that alcohol treatment protected against the downregulation of the sarcoplasmic reticulum Ca^{2+}-ATPase seen in those rats fed a restricted diet (ie, the isocaloric glucose-fed rats). However, it would be highly imprudent to make detailed biochemical comparisons with the ad libitum–fed rats with either of the 2 other groups. This is because the compositions of the solid chow and liquid diets are entirely different with respect to macro- and micronutrients, such as vitamins, minerals, and other essential and nonessential dietary elements. Differences between the rats fed the solid chow ad libitum and the animals on the liquid diets may thus be due to any one of several components. For example, dietary fat, amino acids, vitamins, and minerals and utilizable energy may all alter muscle biochemistry and composition at the gross biochemical or molecular level (for example, see previous works[41-46]). This aspect of alcohol feeding has been the subject of a number of reviews including our own.[20-22] Thus, in comparing the 2 groups of rats fed the liquid diet, we can confidently argue that putative changes within ethanol-exposed skeletal muscle must be due to either ethanol, its metabolites or ethanol-induced metabolic disturbances per se rather than malnutrition. In simple terms, the increase in SERCA1 and Ca^{2+}-ATPase in this present study must have been due to alcohol rather than altered dietary intake.

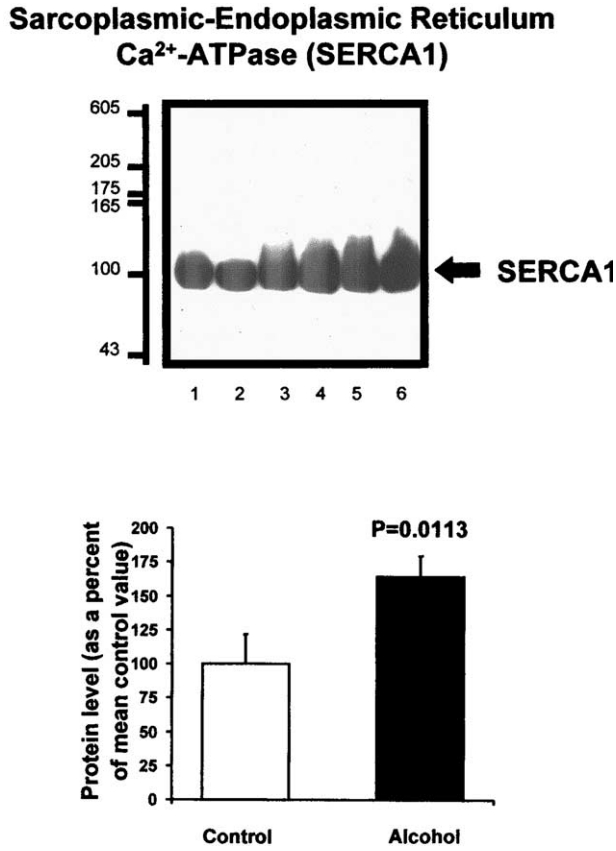

Fig 8. Immunoblot: SERCA1 Ca^{2+}-ATPase in muscle from control and ethanol-fed rats. Shown is an immunoblot labeled with an antibody to the SERCA1 isoform of the Ca^{2+}-ATPase, whereby lanes 1 and 2, 3 and 4, and 5 and 6 are the microsomal fraction isolated from rats representing the control-fed, ethanol-fed, and ad libitum animal groups, respectively. For other details see legend to Fig 3.

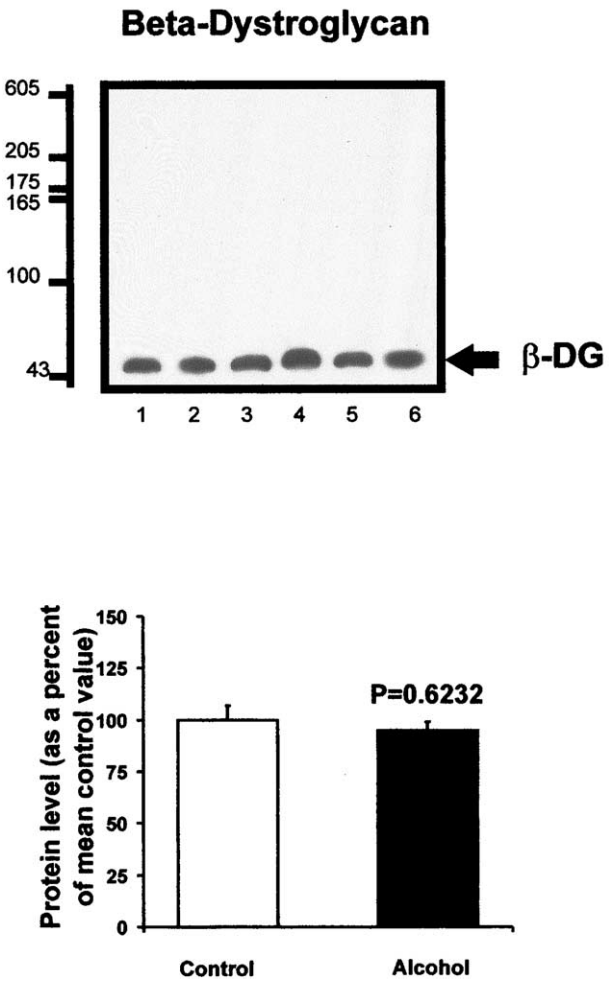

Fig 9. Immunoblot of β-dystroglycan in muscle from control and ethanol-fed rats.Shown is an immunoblot labeled with an antibody to β-dystroglycan, whereby lanes 1 and 2, 3 and 4, and 5 and 6 are the microsomal fraction isolated from rats representing the control-fed, ethanol-fed, and ad libitum animal groups, respectively. For other details see legend to Fig 3.

Membrane Damage in Alcoholism and Other Muscle Disorders

The toxic effects of alcohol appear to be accumulative and alcoholics often exhibit multiorgan involvement such as the heart and liver as well as skeletal muscle.[47] Perturbations in calcium homeostasis appear to be a common pathogenic mechanism. For example, reduced plasma membrane Ca^{2+}-ATPase activities are seen in hepatocyte and neutrophils in alcoholic liver disease[48] and also after carbon tetrachloride damage of liver plasma membranes.[49] These changes have been ascribed to the membrane disordering effects of ethanol.[50,51] Alcoholic myopathology is also postulated to be triggered by alterations in membrane fluidity, ion channel activity, and ion pump regulation, thereby causing, putatively, an overall depression of muscle protein synthesis and muscle contractility.[1,2,35]

We have recently argued that such membrane changes in alcohol myotoxicity may be related to the involvement of free radicals.[2] This is also supported by the observation that the decrease in erythrocyte plasma membrane Ca^{2+}-ATPase due to alcohol exposure can be prevented by supplementation with the anti-oxidant vitamin E.[52] However, studies investigating experimental vitamin E deficiency on skeletal muscle have shown markedly reduced SERCA activity.[53] Other pathologies in skeletal muscle reduce either SERCA Ca^{2+}-ATPase activity or mRNA such as ageing, muscle fatigue, and denervation.[54-57] The directional changes of SERCA in these aforementioned pathologies directly contrast with the observed increase in SERCA1 due to alcohol feeding in the present study. These divergent results may be explained by the fact that the defects in alcohol-exposed muscle are distinctly complex, involving perturbations in protein synthesis, altered protein breakdown, DNA and RNA damage, and protein adduct formation.[1,2,35] Such metabolic abnormalities all have the potential to influence steady-state levels of membrane proteins or their activities either indirectly (for example, via DNA damage) or directly (for example, via inactivation via covalent linkage with acetaldehyde).[1,2,35] However, increases in SERCA1 protein and mRNA in type I fibers are observed in unloading,[58] which is compatible with the results obtained in muscle of alcohol-exposed rats.

In interpreting the significance of our observations, one must distinguish between acute and chronic ethanol exposure. Acutely, alcohol-induced fiber damage has been shown to trigger the leakage of the muscle marker creatine kinase, im-

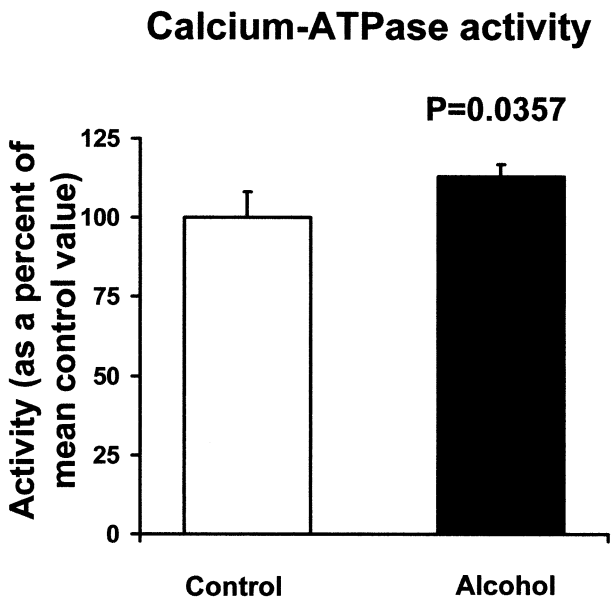

Fig 10. Enzyme activity of the Ca^{2+}-ATPase in muscle from control and ethanol-fed rats. Rats were fed a nutritionally complete liquid diet containing ethanol as 35% of total calories. Controls were pair fed the same diet in which ethanol was replaced by isocaloric glucose. At the end of the study, rats were killed and the muscles dissected out. Histograms pertain to glucose-fed control and ethanol-fed rats and are displayed as the mean ± SEM (n = 8 pairs). *P* values are displayed over the histograms. Detailed analysis of the muscle from chow fed rats was not performed as explained in the main text (Methods and Discussion sections).

plicating impairment in the membrane integrity of skeletal muscle cells.[59] However, plasma creatine kinase activities are not raised in chronic studies, possibly reflecting a component of adaptation or tolerance.[30,60] Such a process of tolerance may be responsible for the upregulation of SERCA1, as opposed to a decrease in SERCA1 seen in most other muscle[54-57,61] and alcohol-related pathologies.[48,49]

Studies on human skeletal muscle biopsies from chronic alcoholics showed reduced myosin Ca^{2+}-ATPase activities.[62] However, it is important to emphasis that a distinction should be made between myofibrillary Ca^{2+}-ATPase and the calcium regulatory enzymes associated with membranes.

The Contribution of Inactivity to Alcoholic Myopathy

There is a need to address the supposition that reduced physical activity in alcoholism may possibly contribute to the myopathy and muscular weakness. It is well known that reduced muscular activity leads to "disuse atrophy." However, the contribution of inactivity as a cause for the myopathy can be discounted for two reasons. First, disuse atrophy is primarily characterized by type I fiber atrophy and/or damage.[63-66] Some studies have shown that disuse atrophy is associated with type II fiber atrophy, but these studies have not dissected out anorexia or other metabolic abnormalities which also cause type II fiber atrophy (for effects of starvation on different fibre-predominant muscles, see Preedy et al[67,68]). Secondly, we have shown that increased activity (via swimming exercise) does not prevent the ethanol-induced type II fiber myopathy in experimental rats.[69] In the aforementioned studies, we used pair-feeding regimens for all treatment groups, which takes account of exercise-induced dietary changes.[69] This contrasts with studies using exercise regimens to prevent the myopathy without primary consideration of nutritional factors.[70]

The Role of Centrally Mediated Depression

Centrally mediated depression may possibly contribute to the phenomena of alcoholic myopathy. Although there is little relevant work on the effects of centrally mediated depression on skeletal muscle calcium regulatory proteins, we can draw analogy with the depressive effects on muscle protein synthesis, which is a characteristic hallmark of alcoholic myopathy. Administration of morphine sulfate and morphine-6-glucuronide depresses skeletal muscle protein synthesis, possibly via respiratory depression.[71] Currently, however, we have no evidence that such comparisons are strictly analogous to the present alcohol-feeding study due to the complexity of alcohol metabolism inducing free radicals, endocrine changes, formation of adducts, and other modes of damage.[1,2,34,35] For example, acute morphine sulfate and morphine-6-glucuronide administration does not alter circulating insulin concentration, although other endocrine changes occur.[71] In contrast, insulin levels increase in response to ethanol.[31]

Implications of the Study

Patients suffering from alcoholic myopathy may lose up to 20% of their musculature.[1,35] Since muscle represents the most abundant mammalian tissue (40% of body weight), chronic alcoholic myopathy has profound implications for the physiology of the whole body.[1,35] In addition, defects in muscle strength and contractility will contribute markedly to increased morbidity and impaired quality-of-life measures in affected subjects.[1,35] It is tempting to speculate that disturbances in SERCA1 may be a contributing factor to this pathology.

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