Alcohol Affects the Skeletal Muscle Proteins, Titin and Nebulin in Male and Female Rats

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ABSTRACT Alcohol myopathy is characterized by decreased protein synthesis and contents resulting in atrophy of muscle fibers. We investigated the effect of alcohol on the cytoskeletal muscle proteins, nebulin and titin. Because women are more susceptible than men to the toxic effects of alcohol, male and female rats were included. Four groups were investigated: alcoholic males, pair-fed males, alcoholic females, pair-fed females. Alcohol consumption per unit body weight was 12.9 g/kg·d, with no difference between males and females. After 10 wk, male and female rats fed alcohol had lower gastrocnemius and plantaris protein and RNA contents (P < 0.001), with no effect on soleus, indicating myopathy of type II fibers. The gastrocnemius was fractionated to measure myofibrillar protein contents. Low percentage SDS-gel electrophoresis was performed to determine myosin heavy chain (MHC), nebulin and titin contents. Alcohol reduced gastrocnemius myofibrillar protein and MHC contents, and the plantaris RNA/protein ratio (P < 0.01). The titin/MHC and nebulin/MHC ratios were unaffected, suggesting a concomitant reduction in titin and nebulin. The decreases in titin and nebulin contents may affect muscle function. An interaction between gender and alcohol was noted for the plantaris RNA/protein ratio (P < 0.025), suggesting a reduced capacity for muscle protein synthesis in females. J. Nutr. 133: 1154–1157, 2003.

KEY WORDS: alcohol • titin • nebulin • protein • muscle

Skeletal muscle myopathy attributed to alcoholism occurs in between one third and two thirds of all chronic alcohol abusers, and is a major cause of morbidity (1,2). Alcoholic myopathy is characterized, in both humans and rats, by a reduction in skeletal muscle protein synthesis, total RNA and protein content, and more specifically, the myofibrillar protein contents (3–8). These changes are confined to muscles rich in type II muscle fibers such as plantaris and gastrocnemius, with soleus (type I fiber predominant) being relatively unaffected (5). Light microscopy with staining for type II fibers confirms that these changes are fiber type specific (9).

The muscular weakness accompanying alcoholic myopathy may therefore be ascribed to a reduction in the contractile apparatus and/or disruption of the contractile process. However, aside from the actual contractile proteins, there are many other proteins intimately involved in the contractile process. Titin (connectin) is a giant filamentous protein that spans from the Z disc to the M line, thereby forming a third filament system of the sarcomere, contributing to passive force development of relaxed muscle fibers, and maintaining the structural integrity of the myofibril during contraction (10). The A band section of titin provides regular binding sites for other sarcomeric proteins, and so may contribute to sarcomere assembly (11,12). The I band section consists of immunoglobulin-like domains, and the PEVK segment, both of which contribute to the extensibility and passive force development of relaxed skeletal muscle fibers when stretched (10). The titin molecule also contains phosphorylation sites, a serine/threonine kinase domain and binding sites for muscle-specific calpain proteases, indicating that it may also play a role in myofibrillar signal transduction pathways (11). Nebulin is an inextensible filamentous giant protein associated with the Z disc, thought to act as a molecular ruler to specify the length of the actin thin filament (13). Nebulin may also act as scaffolding to which actin monomers must join during sarcomere assembly, therefore playing a role in myofibrillar synthesis (14).

This study was designed to quantify changes in the cytoskeletal proteins nebulin and titin attributed to alcohol. We used the new high fat, alcohol feeding regimen, previously shown to cause histological liver lesions exceeding those caused by traditional high carbohydrate diets (15). Clinically, women are more susceptible than men to alcoholic myopathy, cardiomyopathy and liver disease (16,17), so we investigated whether there was any interaction between the effects of alcohol and gender on skeletal muscle biochemistry, to see whether any biochemical basis for this clinical observation could be found.

MATERIALS AND METHODS

Treatment of animals. All rats were treated in accordance with the ethical guidelines provided by the university at which the study was conducted. Wistar rats (Mollegaard, Ejby, Denmark) were individually housed in stainless steel wire cages. There were four groups: 1) male rats consumed the alcohol containing diet ad libitum, 2) male rats pair-fed the control diet, 3) female rats consumed the alcohol containing diet ad libitum, 4) female rats pair-fed the control diet. In alcohol feeding studies, the control rats should always be pair-fed to the alcohol group to control for the anorexia and reduced food consumption of rats fed alcohol (3–8). The mean daily intake of the

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rats fed alcohol was recorded, and weight-matched controls were pair-fed the same amount of the control diet the subsequent day.

The high fat/low carbohydrate liquid feeding regimen was used as described previously (15). This involved modification of the Lieber–DeCarli diet (LD101 and LD101A; Purina Mills, Richmond, IN) described previously (18). A 50% level of energy was provided by the LD101A diet (LD101 in controls), and corn oil was added to increase the fat content from 35 to 44%, with a corresponding decrease in the content of carbohydrate from 11 to 5.5% (or from 47 to 40% in controls). The percentage of energy supplied as ethanol was 34.5% (compared to 36% in the Lieber–DeCarli diet). Casein, vitamins and minerals (technical grade; Sigma, St. Louis, MO) were added to equal the composition of the Lieber–DeCarli diet. In addition, carboxymethylcellulose (CMC, 3.4 g/L; Metsa Specialty Chemicals Oy, Aanekoski, Finland) was added as a stabilizer to increase viscosity. The metabolically inert CMC is widely used as a thickener in a variety of dairy and other food industry products. There was no difference between the male and female rats in the daily consumption of alcohol per unit body weight (40.3% in male rats and 36% in female rats in the daily consumption of alcohol per unit body weight). Alcohol Control alcohol was added as a stabilizer to increase viscosity of the liquid diets, so that body weights are not meaningful (19).

Protein was then subjected to alkali digestion in NaOH (0.3 mol/L) before centrifugation at 33,000 × g, the supernatant was discarded. The protein pellet was then disrupted in a ground-glass homogenizer with an extraction buffer of 10 mL of high ionic strength [potassium dihydrogen orthophosphate, 300 mmol; EDTA, 1.0 mmol; and ATP, 5.0 mmol/L, pH 6.3 (22)]. After centrifugation at 33,000 × g, supernatants containing solubilized “myofibrillar” proteins were decanted. The protein fractions were then processed as described above. Measurement of myofibrillar protein is quantitative, with a fractional recovery of ~100% (3, 21).

Protein fractionation. Homogenates from the above procedures (containing 200 mg tissue) were immediately transferred to 20 mL of low ionic strength buffer, pH 7.0 [imidazole, 10 mmol; KCl, 60 mmol; EGTA, 0.5 mmol; MgCl₂, 4.0 mmol; sodium azide, 1.0 mmol; and dithiothreitol, 1.0 mmol/L plus 0.5% (v/v) Triton X-100] (22). After centrifugation at 33,000 × g, the supernatant was discarded. The protein pellet was then disrupted in a ground-glass homogenizer with 10 mL of high ionic strength [potassium dihydrogen orthophosphate, 100 mmol; dipotassium hydrogen orthophosphate, 50 mmol; KCl, 300 mmol; EDTA, 1.0 mmol; and ATP, 5.0 mmol/L, pH 6.3 (22)]. After centrifugation at 33,000 × g, supernatants containing solubilized “myofibrillar” proteins were decanted. The protein fractions were then processed as described above. Measurement of myofibrillar protein is quantitative, with a fractional recovery of ~100% (3, 21).

**SDS-gel electrophoresis.** Low percentage SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was employed to investigate high-molecular-weight proteins in rat gastrocnemius muscles. Procedures were carried out essentially as described previously (23). Briefly, agarose-strengthened 2.8% polyacrylamide gels with a Laemmli buffer system (24) were prepared to detect titin, nebulin and myosin. Equal amounts of solubilized tissue were loaded in each lane, and the protein contents were then measured spectrophotometrically, by use of a protein assay; Bio-Rad Laboratories GmbH, Munich, Germany). Approximately 100 μg of protein was loaded in each lane, but greater emphasis was placed on equal loading of the lanes. Protein bands were visualized with Coomassie brilliant blue R. Titin and nebulin were previously sequenced for rabbit soleus muscle (molecular weights of 3700 and 800 kDa, respectively), so rabbit soleus was used as a standard to verify that the bands obtained were in fact titin and nebulin (24). Gel images were scanned at a resolution of 600 dpi. Protein bands on scanned gels were analyzed for their optical volume (integrated optical density), using TotalLab software (Phoretix, Newcastle-upon-Tyne, UK). This ensured that gels were assessed objectively, given that ratios were used to express titin and nebulin relative to MHC. Data were expressed as ratios to account for variation between gels. Density ratios between proteins of interest were calculated after constructing calibration curves for myosin, nebulin and titin. The optical volume/mg of total protein was then calculated.

**Tissue processing.** All steps, including homogenization, were carried out at 0–4°C, and all centrifugations were at 2000 × g for 10 min, unless otherwise stated. Muscles were homogenized in ice-cold water, and portions of homogenate containing 200–400 mg tissue were either immediately precipitated with perchloric acid to a final concentration of 0.2 mmol/L or used for protein fractionation (described later).

**Mixed protein and RNA.** After the first precipitation, the acid supernatant was discarded after centrifugation and the protein pellet was then washed twice with perchloric acid (0.2 mmol/L). The pellet was then subjected to alkali digestion in NaOH (0.3 mol/L) before protein estimation by the Biuret reaction (22). The protein was then precipitated, and RNA was measured in the supernatant (3–5).

**Statistical analysis**. Data were expressed as means ± SEM, n = 7–10. Data were compared using two-way ANOVA, with differences of P < 0.05 considered significant.

**RESULTS**

Alcohol decreased the gastrocnemius and plantaris protein and RNA contents in both males and females (Table 1). The

### Table 1

<table>
<thead>
<tr>
<th>Gender</th>
<th>Alcohol</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>Protein</td>
<td>RNA</td>
</tr>
<tr>
<td>Female</td>
<td>Protein</td>
<td>RNA</td>
</tr>
</tbody>
</table>

#### Abbreviations used:
- CMC, carboxymethylcellulose
- MHC, myosin heavy chain

#### Table 1

Muscle RNA and protein contents in male and female Wistar rats fed control and ethanol-containing diets for 10 wk.1,2

<table>
<thead>
<tr>
<th>Gender</th>
<th>Alcohol</th>
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</tr>
</thead>
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<tr>
<td>Male</td>
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<td>RNA</td>
</tr>
<tr>
<td>Female</td>
<td>Protein</td>
<td>RNA</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM, n = 7–10.
2 NS, not significant, P > 0.05.
gastrocnemius myofibrillar protein fraction (Table 1) and MHC (Table 2) were also reduced. Soleus RNA and protein contents of soleus, a type I fiber–predominant muscle, were unaffected by alcohol (Table 1), indicating that the high fat, alcohol diet induced a type II fiber myopathy. Alcohol reduced the plantaris RNA/protein ratio by 5% in males and 13% in females (Table 1), with a significant interaction between alcohol and gender \( (P < 0.025) \). This ratio was unaffected in soleus and gastrocnemius muscles.

Because alcohol decreased gastrocnemius MHC but not the titin/MHC and nebulin/MHC ratios, it also reduced the levels of titin and nebulin (Table 2). Representative gels showing the clear resolution of MHC, titin and nebulin are shown in Figure 1.

**DISCUSSION**

Alcoholic myopathy is characterized by lean tissue wasting, with a reduction in total RNA and protein contents, and specific proteins such as desmin, actin, troponins I and T, and the MHC isoforms (3–8). However, other noncontractile proteins are intimately related to the contractile process such as the sarcosomic cytoskeletal proteins, titin and nebulin. We tested the hypothesis that nebulin and titin would also be affected by ethanol consumption.

**The effect of alcohol on skeletal muscle biochemistry.** The ethanol feeding regimen used resulted in a myopathy of type II fibers, as evidenced by the reduced protein contents in gastrocnemius and plantaris muscles (type II fiber rich), with no effect on soleus (type I fiber predominant). This is well documented in other alcohol feeding studies (6). The myofibrillar protein contents and MHC specifically were also reduced, indicating that the contractile proteins in particular were affected by alcohol, as has been demonstrated in conventional alcohol feeding studies (8). A reduction in tissue synthetic potential was also demonstrated by the decreased RNA/protein ratio in the plantaris muscle.

**The effect of alcohol on titin and nebulin.** Titin contributes to the passive force development of relaxed muscle fibers, maintains the structural integrity of the myofibril during contraction and may also be involved in sarcomere assembly and turnover (10). Titin has been implicated in several disease processes such as limb girdle muscular dystrophy type 2a and cardiac failure (25,26). Nebulin binds tightly to actin and may control the length of actin filaments (13). Genetic mutations in the nebulin gene are associated with nemaline myopathy (27,28). Therefore, titin and nebulin play important roles in normal muscle physiology and in disease.

Titin and nebulin contents were expressed relative to MHC. The titin/MHC and nebulin/MHC ratios were unaffected by alcohol, which indicates that titin and nebulin are reduced proportionately to the decrease in MHC. This reduction in titin and nebulin may decrease the stability of the sarcomere and myofibrils at rest and during contraction, which may impair muscle tension generation and function.

**The effect of gender.** Because women are clinically more susceptible than men to the pathological effects of alcohol (16,17), male and female groups were included to explore any effect of gender on alcohol-induced perturbations in skeletal muscle biochemistry. The RNA/protein ratio in plantaris muscle showed an alcohol/gender interaction, with a greater reduction in females. The RNA/protein ratio is a measure of the tissues capacity for protein synthesis (29). The protein content is a static measure that gives little insight into rates of synthesis and proteolysis. The decreased protein content in alcoholic myopathy is attributed to reduced rates of protein synthesis. This is thought to be under both translational and transcriptional control, with a reduction in ribosomal RNA.

**TABLE 2**

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>Control</th>
<th>Female</th>
<th>Control</th>
<th>Two-way ANOVA (P-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MHC (optical volume/mg protein)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alcohol</td>
<td>1.15 ± 0.17</td>
<td>1.64 ± 0.13</td>
<td>1.50 ± 0.16</td>
<td>1.96 ± 0.19</td>
<td>NS &lt;0.01 NS</td>
</tr>
<tr>
<td>Control</td>
<td>1.32 ± 0.18</td>
<td>1.60 ± 0.14</td>
<td>1.40 ± 0.18</td>
<td>1.80 ± 0.19</td>
<td>NS NS NS</td>
</tr>
<tr>
<td><strong>Nebulin/MHC</strong></td>
<td>0.0630 ± 0.0108</td>
<td>0.0515 ± 0.0062</td>
<td>0.0625 ± 0.0217</td>
<td>0.0311 ± 0.0052</td>
<td>NS NS NS</td>
</tr>
<tr>
<td><strong>Titin/MHC</strong></td>
<td>0.0543 ± 0.0037</td>
<td>0.0489 ± 0.0048</td>
<td>0.0733 ± 0.0262</td>
<td>0.0558 ± 0.0093</td>
<td>NS NS NS</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM, \( n = 7–10 \).

2 NS, not significant, \( P > 0.05 \).

LITERATURE CITED

**FIGURE 1** A 2.8% SDS–polyacrylamide gel showing separation of titin, nebulin and MHC bands for gastrocnemius muscle of control male and female rats. To verify the identity of the bands labeled titin and nebulin, rabbit soleus was run as a standard because titin and nebulin have both been sequenced in this muscle.