Effect of Prolonged Alcohol Administration on Calcium Transport in Heart Muscle of the Dog

By Richard J. Bing, Harald Tillmanns, Jean-Marie Fauvel, Keith Seeler, and James C. Mao

ABSTRACT

The effect of prolonged administration of alcohol on calcium binding and uptake by sarcoplasmic reticulum and mitochondria and on respiratory function of mitochondria was investigated in heart muscle of dogs. Dogs were paired and maintained with and without alcohol for 6 months; alcohol was administered by adding it to drinking water and food with vitamin supplements. Measurements were made after alcohol had been temporarily withheld for 2 days. Prolonged alcohol ingestion resulted in a decline in calcium binding and uptake by sarcoplasmic reticulum and mitochondria, suggesting a diminished affinity of the reticular and mitochondrial membranes for calcium ions. The endogenous calcium content of mitochondria and sarcoplasmic reticulum decreased. Prolonged alcohol administration failed to alter cardiac contractility, although contraction and relaxation tended to diminish following the administration of angiotensin. The results illustrate that one link in the regulation of the state of contraction or relaxation involving myofibrillar calcium transport is weakened in dogs maintained on alcohol for prolonged periods of time.

KEY WORDS calcium binding and uptake mitochondrial respiration sarcoplasmic reticulum alcohol and cardiac contractility excitation-contraction coupling

In a previous report from this laboratory (1), observations were presented suggesting that one primary myocardial aberration which occurs after prolonged ingestion of alcohol is a biochemical malfunction of mitochondria (1). This malfunction is reflected by the diminished activities of various intramitochondrial and extramitochondrial enzymes, especially isocitrate dehydrogenase. In addition, mitochondrial oxygen consumption and respiratory control indexes in dogs given to alcohol are diminished. Observation of the disturbed mitochondrial function in animals subjected to prolonged ingestion of alcohol suggests that the disturbances might be accompanied by changes in calcium metabolism. This assumption is based on the work of Lindenmayer et al. (2), who found a close correlation between the impairment of electron transport and the calcium uptake of mitochondria. Furthermore, Sordahl et al. (3) have established the fact that, although the primary function of mitochondria—oxidative phosphorylation—is sustained until very late in myocardial failure, other energy-linked functions such as calcium transport are rapidly affected.

The present study is primarily concerned with the effects of prolonged (6 months) ingestion of alcohol in dogs on calcium binding and uptake by sarcoplasmic reticulum and mitochondria and with the possible relationship of these changes to alterations in mitochondrial respiration and cardiac contractility.

Methods

Experiments were carried out on 14 mongrel dogs. The dogs were divided into two groups: one group (C) served as controls and the other (A) received alcohol. Each dog also served as its own control. The data collected in the dogs before alcohol was administered (group A1) were compared with those collected in the same dogs after prolonged administration of alcohol for 6 months (group A2). The same procedure was followed for the dogs that were not given alcohol (groups C1 and C2). However, some analyses (mitochondria, sarcoplasmic reticulum, and high-energy phosphates) required large amounts of cardiac tissue which could be obtained only at the end of the observation period when the whole heart was available. In these instances, groups C2 and A2 were compared.

Each dog that received alcohol was paired with a control dog of similar body size and weight; the dogs were maintained for the same period under identical conditions except that alcohol was withheld in one group. The diet consisted of 2,000 calories of meat, meat by-
products, and vitamin supplements. The total caloric intake in both the alcohol-fed and the control group was the same; the carbohydrate fraction of the diet was lowered in the alcohol-fed dogs to compensate for the calories derived from alcohol, but the protein and fat fractions were held constant. Alcohol (400 ml of a 25% solution) was added to the food and the drinking water, and the dogs consumed this amount of alcohol within 24 hours. Blood alcohol levels were determined on several dogs at various time intervals. As reported previously (1), alcohol levels of 150–225 mg/100 ml blood were achieved; peak alcohol levels occurred 3 hours after ingestion. Studies on dogs given alcohol were carried out 2 days after alcohol was withheld.

HIGH-ENERGY PHOSPHATES

Creatine phosphate and adenosine-5-triphosphate (ATP) were determined enzymatically according to the procedures of Fawaz and Manoukian (4) and Bergmeyer (5).

ISOLATION OF MITOCHONDRIA AND SARCOPLASMIC RETICULUM

The experimental techniques used to isolate mitochondria and sarcoplasmic reticulum were similar to those described previously (1). Mitochondria were isolated from the left ventricular myocardium according to the procedures of Fawaz and Manoukian (4) and Bergmeyer (5). Mitochondria and sarcoplasmic reticulum were similar to those described previously (1). Mitochondria were isolated from the left ventricular myocardium according to the procedure of Lindenmayer et al. (2). Sarcoplasmic reticulum was isolated from the supernatant fluid obtained from the first crude mitochondrial pellet (7,700 g for 10 minutes), using a modification of the procedure of Harigaya and Schwartz (6). The supernatant fluid was centrifuged at 17,300 g for 10 minutes; this pellet was termed the light mitochondrial fraction. The supernatant fluid was then centrifuged at 37,000 g for 30 minutes. The resulting pellet was quickly washed in a medium containing 20 mM Tris-maleate, pH 6.8, with 50 mM KC1 and 0.5% bovine serum albumin (BSA). BSA appears to be important for isolation of mitochondria and sarcoplasmic reticulum preparations. Therefore, mitochondria and sarcoplasmic reticulum were prepared from the same tissue, and BSA was used throughout the preparations.

MARKER ENZYME ASSAY

All fractions obtained from the purification scheme were analyzed using the following marker enzymes for checks on purity and yield: an azide-insensitive calcium (Ca++)-activated adenosine triphosphatase (ATPase) for sarcoplasmic reticulum and succinate cytochrome C reductase for mitochondria (7, 8). The ATPase activity was determined by measuring the inorganic phosphate (P,) liberated from ATP according to the method of Fiske and Subbarow (9).

MEASUREMENT OF MITOCHONDRIAL RESPIRATION

The measurement of mitochondrial respiration was accomplished within 1 hour after isolation with a vibrating platinum oxygen electrode (Oxygraph, Gilson Medical Electronics) as described previously (1).

CALCIUM BINDING AND UPTAKE BY MITOCHONDRIA AND SARCOPLASMIC RETICULUM

A slightly modified Millipore filter method was used to measure calcium binding (6). The determinations of calcium binding by sarcoplasmic reticulum and mitochondria were carried out in ml (total volume) of solutions containing 50 μM CaCl_2 with 0.045 μg of 45Ca, varying concentrations of EGTA (ethyleneglycol bis[b-aminoethyl]ether]-N',N'-tetraacetic acid), 120 mM KCl, 5 mM MgCl_2, 20 mM Tris-maleate buffer, pH 6.8, 5 mM Tris-ATP, and 200–400 μg/ml of membrane protein (either sarcoplasmic reticulum or mitochondria). Calcium uptake of sarcoplasmic reticulum was determined when 2.5 mM Tris-oxalate was added to the medium described in the preceding paragraph and the calcium concentration was increased to 100 μM. The protein concentration in sarcoplasmic reticulum was decreased to between 20 and 40 μg/ml. Calcium uptake by mitochondria was determined in the presence of 4 mM P, and 5 mM Tris-succinate.

The reaction of calcium binding and uptake was started by the addition of sarcoplasmic reticulum or mitochondria at 37°C. It was stopped by filtration through a Millipore filter (HAWP 02500, HA 0.45μ, white, 25 mm). The ultrafiltrate was suspended in a medium containing 0.1% ethyleneglycol monooxyether, 10% naphthalene, 0.4% 2,5-diphenyloxazole in dioxane; the 45Ca content was determined in a liquid scintillation counter. Calcium binding and uptake were estimated from the radioactivity of the filtrate by subtracting the specific activity in the filtrate from that in the unfiltered reaction mixture.

ATPase ACTIVITY

Calcium binding and uptake were associated with a calcium-activated magnesium-dependent ATPase (Ca++, Mg++-ATPase), the activity of which was measured from the P liberated from ATP (9). The protein concentration of sarcoplasmic reticulum and mitochondrial preparations was determined by the Biuret method (10).

ENDOGENOUS CALCIUM CONTENT OF SARCOPLASMIC RETICULUM AND MITOCHONDRIA

Preparations were measured with an atomic absorption spectrophotometer (model 107, Perkin-Elmer Corp.) in the presence of 1% lanthanum chloride and 0.4N perchloric acid (11).

HEMODYNAMIC STUDIES

Hemodynamic studies were performed in 12 dogs anesthetized with sodium pentobarbital (30 mg/kg, iv). These studies included determinations of left ventricular end-diastolic pressure and the first derivative of the left ventricular pressure rise. The method of Cohn and co-workers was employed to determine the rates of ventricular relaxation (12). These workers measured the rate of ventricular pressure fall during relaxation, since this negative dP/dt appears to be related to both the intrinsic contractility of the ventricle and the volume of the heart when relaxation commences. Measurements were performed with a tip manometer and were repeated after 3 minutes of angiotensin infusion (1.91 μg/min). Recordings were made on an Electronics-for-Medicine recorder.
Statistical analyses were performed using Student’s t-test (13).

Results

HIGH-ENERGY PHOSPHATES: RESPIRATORY FUNCTION OF MITOCHONDRIA

In essence, the results of this study are similar to those published previously (1), except that in the present study the difference between high-energy phosphates in the control group and the experimental group was very small (creatine phosphate: group C, 9.3 ± 0.5 μmoles/g tissue and group A, 8.9 ± 0.2 μmoles/g tissue; ATP: group C, 4.9 ± 0.1 μmoles/g tissue and group A, 5.1 ± 0.2 μmoles/g tissue). As reported previously (1) mitochondria from the hearts of dogs given alcohol (group A) exhibited below normal respiratory rates and depressed respiratory control indexes with little change in the efficiency of ATP production compared with control dogs (group C) (Table 1). The protein content of mitochondria was maintained at 1.6 mg/assay. The total protein yield of mitochondria averaged 5.8 mg/g wet tissue. Respiratory function and calcium transport of mitochondria were both impaired (Table 1 and Fig. 1).

CALCIUM BINDING AND UPTAKE OF SARCOPLASMIC RETICULUM AND MITOCHONDRIA

Figure 2 shows a marked reduction in calcium binding by the reticular fraction isolated from the hearts of dogs given alcohol (group A). The difference in calcium binding between groups A and C was statistically significant (P < 0.025). Figure 1 shows the calcium uptake by the reticular fractions of groups C and A; the diminution of calcium uptake in group A was significant.

Calcium binding by mitochondria from hearts of control dogs (group C) was significantly higher than that from hearts of dogs given alcohol (group A) (Fig. 2). Changes in calcium uptake by mitochondria were similarly altered (Fig. 1).

Table 1

Mitochondrial Respiratory Function in Heart Muscle of Dogs Maintained with and without Alcohol

<table>
<thead>
<tr>
<th></th>
<th>Group C</th>
<th></th>
<th>Group A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinate</td>
<td>RCI</td>
<td>ADP:O</td>
<td>QO₂</td>
</tr>
<tr>
<td></td>
<td>8.5 ± 1.1</td>
<td>1.9 ± 0.02</td>
<td>112.3 ± 6.4</td>
</tr>
<tr>
<td>Glutamate-oxaloacetate</td>
<td>20.9 ± 1.0</td>
<td>3.3 ± 0.1</td>
<td>134.1 ± 5.2</td>
</tr>
<tr>
<td>Pyruvate-malate</td>
<td>19.3 ± 1.2</td>
<td>3.5 ± 0.1</td>
<td>91.1 ± 2.5</td>
</tr>
<tr>
<td>β- Hydroxybutyrate</td>
<td>17.7 ± 0.7</td>
<td>3.5 ± 0.1</td>
<td>97.5 ± 4.9</td>
</tr>
</tbody>
</table>

All values are means ± se for seven dogs. C = control dogs after 6 months, A = dogs given alcohol for 6 months, RCI = respiratory control index, ADP:O = ratio of nanomoles of phosphorylated adenosine diphosphate to nanoatoms of oxygen, QO₂ = oxygen consumption in nanoatoms O₂/min mg⁻¹ mitochondrial protein.

* P < 0.0025.

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Calcium binding by sarcoplasmic reticulum and mitochondria from heart muscle of dogs maintained with and without alcohol (groups A, and C, respectively). Calcium binding was measured at 37°C by the Millipore filter method. Values are means ± SE calculated from an average of seven dogs. Open circles - group C, and solid circles - group A, for sarcoplasmic reticulum; open triangles - group C, and solid triangles - group A, for mitochondria.

calcium transport in group A, was significantly diminished.

**ENDOGENOUS CALCIUM CONTENT OF SARCOPLASMIC RETICULUM AND MITOCHONDRIA**

The endogenous calcium content of sarcoplasmic reticular and mitochondrial fractions isolated from heart muscle of control dogs (group C,) was compared with that from heart muscle of dogs given alcohol (group A,). The amount of calcium in sarcoplasmic reticular and mitochondrial fractions isolated from dogs in group C, was 10.9 ± 0.7 nmoles/mg protein and 9.0 ± 0.8 nmoles/mg protein, respectively. In group A, the values were 7.6 ± 0.5 nmoles/mg protein and 5.8 ± 1.1 nmoles/mg protein, respectively. The data illustrate that the endogenous calcium content of both sarcoplasmic reticulum and mitochondria was significantly decreased in group A (P < 0.025).

**MARKER ENZYME ASSAY**

The results of the marker enzyme assays showed that the sarcoplasmic reticular fraction was contaminated with mitochondria by 1.4% for groups C, and A,. Mitochondrial fractions were contaminated with sarcoplasmic reticulum by 0.8% and 0.6% in groups C, and A,, respectively.

**HEMODYNAMIC STUDIES**

Table 2 illustrates that left ventricular end-diastolic pressure decreased from 5.6 to 2.5 mm Hg and that maximum rate of rise of left ventricular pressure (dP/dt max) increased from 2281 mm Hg/sec to 2829 mm Hg/sec in dogs maintained on alcohol (group A,). These changes were significant. The maximum rate of fall of left ventricular pressure (negative peak dP/dt) was not significantly altered by the action of alcohol.

During infusion of angiotensin, left ventricular end-diastolic pressure increased in all groups. In dogs given alcohol (group A,), angiotensin infusion resulted in a decline in dP/dt max; this decline, however, was not statistically significant. Because of the significant difference between values for dP/dt max in group A, compared with group A, (Table 2), the change in response to angiotensin in group A, lost its importance. As Table 2 demonstrates, administration of angiotensin resulted in different degrees of change in negative peak dP/dt between control and experimental dogs. The percent change in negative peak dP/dt was negative.

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**TABLE 2**

**Hemodynamic Studies in Six Dogs Maintained with and without Alcohol**

<table>
<thead>
<tr>
<th></th>
<th>Before angiotensin</th>
<th>After angiotensin</th>
<th>Before angiotensin</th>
<th>After angiotensin</th>
<th>Before angiotensin</th>
<th>After angiotensin</th>
<th>% Change after angiotensin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Left ventricular end-diastolic pressure (mm Hg)</td>
<td>dP/dt max (mm Hg/sec)</td>
<td>Peak negative dP/dt (mm Hg/sec)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group A,</td>
<td>5.6 ± 0.4</td>
<td>13.1 ± 2.4</td>
<td>2281 ± 232</td>
<td>2498 ± 245</td>
<td>3202 ± 596</td>
<td>3074 ± 452</td>
<td>– 4 ± 11</td>
</tr>
<tr>
<td>Group A,</td>
<td>2.3 ± 0.7</td>
<td>11.3 ± 2.1</td>
<td>2829 ± 231</td>
<td>2491 ± 131</td>
<td>3720 ± 407</td>
<td>3274 ± 498</td>
<td>–12 ± 8</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.0025</td>
<td>&lt;0.01</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Group C,</td>
<td>4.8 ± 1.1</td>
<td>15.7 ± 3.3</td>
<td>2490 ± 246</td>
<td>2263 ± 85</td>
<td>2798 ± 337</td>
<td>2692 ± 314</td>
<td>– 7 ± 8</td>
</tr>
<tr>
<td>Group C,</td>
<td>4.3 ± 1.0</td>
<td>9.5 ± 0.7</td>
<td>2414 ± 244</td>
<td>214 ± 166</td>
<td>3094 ± 753</td>
<td>3218 ± 417</td>
<td>+ 4 ± 7</td>
</tr>
<tr>
<td>P</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

A, = control data on dogs later given alcohol, A, = dogs given alcohol for 6 months, C, = control data on dogs not given alcohol, C, = control dogs after 6 months, dP/dt max = maximum rate of rise of left ventricular pressure, peak negative dP/dt = maximum rate of left ventricular pressure fall, and NS = not significant.

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ALCOHOL AND CALCIUM TRANSPORT

for group C₁ (−7%) but became positive for group C₂ (+4%). In contrast, negative peak dP/dt became more negative after exposure to alcohol (group A₁, −4% vs. group A₂, −12%). However, scatter of the data was so great that the differences between the groups were not statistically significant (Table 2).

Discussion

The present study was primarily concerned with calcium binding and uptake by sarcoplasmic reticulum and mitochondria of control dogs and dogs given alcohol for 6 months. Data on mitochondrial respiration and cardiac dynamics were included to relate disturbances in calcium transport to possible changes in contraction and relaxation, although these subjects have been studied previously (1).

Excitation-contraction coupling and relaxation in heart muscle are generally considered to be interactions between myofibrillar proteins—actin and myosin—and an energy-dependent calcium movement. An action potential of the depolarizing heart muscle cell travels along the transverse sarcotubular system; it can release calcium from the sarcoplasmic reticulum which can, in turn, result in muscular contraction. Relaxation occurs as the intracellular free calcium is taken up by the sarcoplasmic reticulum through ATP-dependent mechanisms. Recently, it has been suggested that mitochondria might participate in the physiopathology of myocardial failure (2, 3, 16-21).

Previous studies (1) have shown that administration of alcohol to dogs for 14 weeks affects mitochondrial respiration without diminishing cardiac contractility. Infusion of angiotensin resulted in a decline in dP/dt max in dogs given alcohol (group A₁) but not in the same dogs prior to administration of alcohol (group A₂); the decline was only of border line significance (0.1 > P > 0.05).

Mitochondria from the hearts of dogs given alcohol (group A₁) exhibited depressed calcium binding and uptake in the presence of succinate-linked oxidation (Figs. 1 and 2). Similar results were obtained in the sarcoplasmic reticulum from the hearts of the same dogs (Figs. 1 and 2).

Because hearts of dogs given alcohol have diminished calcium binding and uptake by sarcoplasmic reticulum and mitochondria, disturbances in cardiac relaxation could occur. Calculations based on the method of Cohn and co-workers (12), who used negative peak dP/dt as an index of the relaxation process, indicated that relaxation tended to diminish during angiotensin infusion. However, because of considerable scatter in the data, the values were not statistically significant (Table 2).

Several possibilities might explain the effect of alcohol on calcium transport in heart muscle. Disturbed respiratory function of the mitochondria could be a contributory factor, since calcium binding and uptake by mitochondria depend partially on the rate of substrate oxidation and on energy-dependent processes. A deficient ability of mitochondria to take up calcium has also been found by Sordahl et al. (3) in the failing hearts of rabbits.

There are two possible explanations for the fact that a diminution in calcium binding and uptake after alcohol ingestion does not diminish myocardial contractility. First, the changes in calcium transport were not as severe as those found after myocardial infarction, congestive heart failure, or cardiomyopathy (2, 3, 20, 22-24). Second, in both acute myocardial ischemia and hereditary cardiomyopathy of the hamster, the contractile elements are morphologically involved, and the mitochondrial lesions are only part of the general process of myocardial reaction.

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