PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 164, 415–420 (1980)

Mechanical and Subcellular Function of Rat Myocardium during Chronic Ethanol Consumption (40887)[1]

ANDREA H. HASTILLO, JAMES POLAND, AND MICHAEL L. HESS

Departments of Medicine and Physiology, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298

Abstract. Hemodynamic, mechanical, and subcellular function in a rat model of chronic ethanol consumption (A) and pair-fed control rats (C) were evaluated over a 17-week course. Between 5 and 17 weeks, isovolumetric peak systolic pressure did not change (C = 260 ± 20 mmHg; A = 265 ± 35 mmHg; $p > 0.1$). At 17 weeks myocardial contractility as evaluated by (dP/dt-max)/32 × LVP began to decline in the A rats (V_{max} = 1.46 ± 0.12, r = 0.89; C = 1.560 ± 0.09, r = 0.95) but this is not statistically significant. Neither the velocity of calcium uptake by the isolated sarcoplasmic reticulum (SR) nor the whole heart homogenate (H) was different between C and A rats during the 17-week period and the estimated quantitative content of SR/g Heart did not change (at 17 weeks: mg SR/g Heart = 0.15, C and A). Utilizing Triton X-100 purification of cardiac myofibrils, Mg^{2+}-dependent, Ca^{2+}-stimulated myofribrillar ATPase activity began to decline at 11 weeks (A = 80% of control) and continued to decline at 17 weeks (A = 72% of control). These data suggest that a depression of contractile protein function may be an early alcohol-induced derangement in myocardial contractility that precedes the onset of significant mechanical dysfunction.

Clinicians have long noted a strong association between alcoholism and congestive heart failure (1). In an attempt to further understand this clinical syndrome of alcoholic cardiomyopathy (2), several animal models of both acute and chronic ethanol administration have been developed and studied (3–9). It is generally agreed that both acute (5) and chronic (6, 8) ethanol administration can depress cardiac function. The precise subcellular dysfunction that leads to this depression of cardiac contractility has not been explicitly clarified. Although separate instances of depressed mitochondrial function (7, 9–11), sarcoplasmic reticulum function (3, 12), impaired fatty acid oxidation (13), and myofibrillar function (6) as well as histological changes (3, 14) of acute and chronic ethanol administration have been described; few clearly integrated studies have been generated. One such study by Sarma *et al.* (8) in a canine model following 29 months of ethanol administration demonstrated a decrease in myocardial contractility as measured by force–velocity curves and a significant, but small, decrease in sarcoplasmic reticulum calcium uptake. They then speculated that alcohol may induce a depression of contractile protein function.

The present study was designed to correlate mechanical function and the subcellular function of the sarcoplasmic reticulum and the myofibrils during the course of chronic ethanol administration in an attempt to generate a more complete understanding of the pathogenic sequence of alcoholic cardiomyopathy.

Materials and methods. Male Sprague–Dawley rats weighing about 150 g were provided with 23% ethanol for drinking and Purina rat chow *ad libitum*. Age-matched controls received similar volumes of rat chow and a continuous supply of drinking water. Acute effects of ethanol were avoided by allowing 2 days of withdrawal from alcohol before the rats were sacrificed.

[1] Supported by a Grant-in-Aid from the American Heart Association/Virginia Affiliate and in part from funds contributed by the American Heart Association/Richmond Area Chapter and HL 24917-01. This work was performed during the tenure of an Established Investigatorship from the American Heart Association to M.L.H.

0037-9727/80/080415-06$01.00/0

Copyright © 1980 by the Society for Experimental Biology and Medicine.
All rights reserved.

Left ventricular hemodynamics were evaluated by anesthetizing each rat with urethane (1.2 mg/g) intraperitoneally, endotracheal intubation, and positive pressure ventilation and exposing the heart with a mid-line incision. A 20-gauge needle attached by polyethylene tubing to a Statham strain-gauge was sutured to the left ventricle with the bevel of the needle in the left ventricular chamber.

The ascending aorta was isolated in preparation for aortic clamping. ECG and left ventricular *dP/dt* were recorded simultaneously with left ventricular pressure by a Grass polygraph before and after aortic clamping. The frequency response of the pressure transducer and Grass polygraph (minimal 60 Hz) was quite adequate to record pressure changes at heart rates found in rats. A Wise Electric, Richmond, Va. differentiator was used to determine *dP/dt* from the pressure recording. Care was taken to include pressures recorded early in the 10-sec clamping period and during a sequence of normal sinus rhythm. The rats to be used for functional analysis of subcellular components of the myocardium were sacrificed by cervical fracture. The ventricular tissues from control and alcoholic rats were separately pooled in iced saline, washed, blotted dry, diced, weighed, and added to 4 vol of extraction solution (0.3 *M* sucrose, 0.01 *M* imidazole, pH 7.0). The pooling of about seven hearts was necessary to obtain enough tissue for isolation of workable quantities of sarcoplasmic reticulum (SR) and myofibrils. However, this pooling made impossible a statistical treatment of the individual hearts' characteristics. Homogenization was carried out for 20 sec in a Sorvall Omnimixer and an aliquot saved to be used in studies of whole homogenates. The remaining homogenate was centrifuged at 800*g*-max for 20 min to remove myofibrils and cell debris. Further purification of the myofibrils was done by the procedure described by Solaro *et al.* (15). This method utilizing Triton X-100 virtually eliminates contamination by sarcoplasmic reticulum, mitochondria, and sarcolemma and has recently been independently reviewed by Sheuer and Bhan (16) as yielding the "closest to the true physiologic ATPase activity" of the contractile proteins. The supernatant was centrifuged at 8000*g*-max for 25 min to remove mitochondria and the supernatant from this spin was filtered through cheese cloth and recentrifuged at 32,800*g*-max for 40 min to obtain a pellet of high SR concentration. This SR pellet was resuspended in 0.6 *M* KCl and 20 m*M* Tris maleate at pH 6.8 and centrifuged at 32,000*g*-max for 30 min. The final SR pellet was suspended in 20 m*M* Tris maleate, pH 6.8, and 50 m*M* KCl. The protein concentration of this SR preparation was determined by the method of Lowry (17).

The ability of the SR vesicles, whether in whole homogenates or in isolated SR preparations, to sequester calcium was determined by adding a spectrum of concentrations of either whole homogenate or isolated SR to a bath solution at 37°C and containing ^{45}Ca. *K*-Oxalate (10 m*M*) was added to the medium to amplify the capacity of the SR for calcium and *Na*-azide (10 m*M*) was added to inhibit calcium uptake by mitochondria (18). The concentration of free calcium was set, by addition of $CaCl_2$ (0.64 m*M*) and EGTA buffer (1.0 m*M*), at an initial concentration of 1.7 μM. The incubation medium contained 104 m*M* KCl, 10.0 m*M* imidazole (pH 7.0), 8.0 m*M* creatine phosphate, 5.0 m*M* $MgCl_2$, and 5.0 m*M* ATP. Aliquots of the incubation media were removed and filtered through a Millipore filter (0.45 μm). The filtrate was then counted in a scintillation counter.

The velocity of calcium uptake by SR was determined in a similar manner by using an incubation medium with neither EGTA nor creatine phosphate. $CaCl_2$ was 0.09 m*M*. Aliquots were taken at 60 sec, filtered through Millipore filters, and then counted.

Calcium-stimulated, Mg^{2+}-dependent myofibrillar ATPase activity was determined by the rate of release of inorganic phosphate in an incubation medium containing 2 m*M* ATP, 2 m*M* $MgCl_2$, 50 m*M* KCl, 20 m*M* imidazole (pH 7.0) and 10 m*M* *Na*-azide. Similar determinations were made with 1.6 m*M* EGTA added to the medium to set the free calcium concentra-

tion at less than 10^{-7} in order to determine basal ATPase activity. The amount of inorganic phosphate released in the presence of EGTA was subtracted from the amount released in the absence of EGTA to yield the calcium-dependent ATPase.

Results. The rats maintained on chronic alcohol drank on the average 12 ml of 23% ethanol per day while the control rats drank water *ad libitum*. This rate of ethanol consumption is approximately the same as the rate of ethanol metabolism by the rats. Random serum alcohol determinations were in the range of 90–100 mg%. The caloric intake was lower in both the alcohol group and the pair-fed controls than is normally seen in rats fed *ad libitum*. Examination of the rats at the same time of sacrifice demonstrated no significant difference in body weights and general appearance, and no indication of congestive heart failure (pleural effusions, ascites, or higher heart weights).

Figure 1 presents the hemodynamic data from the time-matched, pair-fed controls (C) and the chronic ethanol group (A). During the 17-week study protocol, no change in peak systolic pressures after aortic clamping could be detected in either group. In both the A and C groups, peak systolic pressures increased by at least 200% with aortic clamping and none demonstrated a decrease in systolic pressure during the 10 sec the aorta was clamped. As expected from these data, both C and A

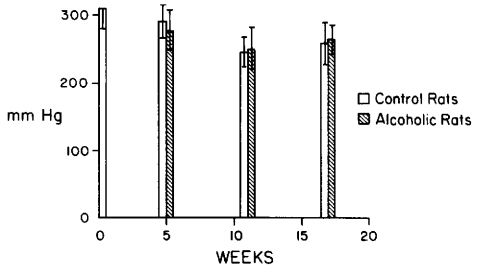

FIG. 1. Isovolumetric peak systolic pressure determined at 0, 5, 11, and 17 weeks in pair-fed controls (n = 4) and the ethanol group (n = 4) 48 hr after the cessation of ethanol. Each bar represents the mean of 8–10 consecutive beats. The vertical line represents ±1 SEM.

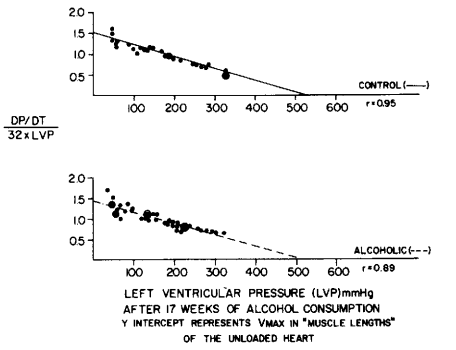

FIG. 2. Derived force–velocity curve at 17 weeks in the pair-fed control (n = 4) and the ethanol group (n = 4) 48 hr after the cessation of ethanol (C: n = 25, y intercept = 1.56 ± 0.09, r = 0.95, y at x of 300 = 0.6446; A: n = 34; y intercept = 1.46 ± 0.12, r = 0.88, y at x of 300 = 0.629) $p > 0.1$.

groups demonstrated similar dP/dt-max values. Figure 2 presents a comparison of the contractile state of the myocardium following 17 weeks of chronic ethanol consumption. Utilizing the formula, $(dP/dt)/32 \times$ LVP (19), as an index of contractility, modified force–velocity curves were constructed for the C and A rat hearts. Neither the X intercept, which represents P_0, nor the Y intercept, which represent V_{max} in muscle-lengths/sec, is significantly different between C and A rats.

A possible site of action at a subcellular level for a potential defect in cardiac contractility could be at either the sarcoplasmic reticulum or the myofibrils since both of these sites have been incriminated in other models of acute and chronic heart failure (20). Figure 3 presents a composite evaluation of the sarcoplasmic reticulum of both groups after the 17-week protocol period. This presentation represents an application of the method of Solaro and Briggs (21) for the estimation of the functional capability of the sarcoplasmic reticulum in normal canine myocardium. This figure demonstrates that (a) there was no significant difference between the ratio of the velocity of calcium uptake of isolated SR and whole homogenates between the two groups after the 17-week period, and (b) the ratio of functional SR/g heart (22) is not significantly different between the two groups,

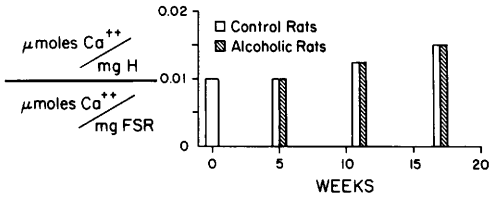

FIG. 3. Quantitative rates of calcium uptake from the whole heart homogenate (μmoles Ca^{2+}/g H) and the isolated sarcoplasmic reticulum (μmoles Ca^{2+}/mg FSR) at 0, 5, 11, and 17 weeks 48 hr after the cessation of ethanol consumption. Each bar represents the pooled heart data from seven hearts in each group. See Methods for experimental procedures.

indicating that chronic ethanol consumption does not interfere or dilute out the amount of functional SR as seen in other cardiomyopathic processes (20, 21). Thus, it would appear that in the rat the sarcoplasmic reticulum calcium transport system remains intact and is thus not the site of initial dysfunction in the development of alcoholic cardiomyopathy.

In contrast, Fig. 4 presents the Triton X-100-purified, calcium-stimulated, magnesium-dependent ATPase data over the 17-week course. After 10 and 17 weeks of ethanol consumption, the calcium-dependent myofibrillar ATPase activity was depressed by 20 and 28%, respectively. Since no difference in calcium-independent ATPase (basal) activity was found, in the presence of EGTA, this represents a true depression of myofibrillar ATPase activity

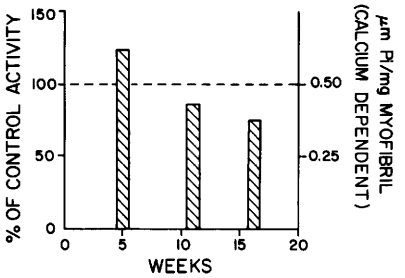

FIG. 4. Ca^{2+}-dependent, Mg^{2+}-stimulated myofibrillar ATPase activity of Triton X-100-purified cardiac myofibrils at 0, 5, 11, and 17 weeks 48 hr after the cessation of ethanol consumption. Each bar represents the pooled heart data from seven hearts in each group. See Methods for experimental procedures.

and incriminates contractile protein dysfunction in "alcoholic cardiomyopathy."

Discussion. In studies by Maines and Aldinger (6) a decrease in myocardial contractility was noted in Sprague–Dawley rats after a similar 16-week period of 25% ethanol ingestion and progressively decreased further over the 32 weeks of their study. However, their findings are similar to those following acute ethanol administration which include bradycardia, a decrease in contractility, and a decrease in tension development (5, 23). The major difference with the study of Maines and Aldinger may be the elapsed time between withdrawal of ethanol and the measurement of contractility. By permitting 2 days between the withdrawal of ethanol and the studies, we avoided the acute effects of ethanol on the myocardium and thus were able to more clearly study any underlying cardiomyopathy.

Other animal models of alcoholic cardiomyopathy have been reported to incriminate a direct effect of chronic ethanol toxicity on the myocardium. Burch *et al.* (4) have reported that in mice, after only 8 to 10 weeks of ethanol administration, electron microscopic changes involve the T-tubule system, the sarcoplasmic reticulum, and the myofibrils. They did report that these electron microscopic abnormalities were variable and subcellular function was not evaluated. In the dog model of chronic ethanol consumption, Bing *et al.* (3) first reported a depression of the sarcoplasmic reticulum after 6 months of ethanol administration with no decrease in mechanical performance noted. In a subsequent study from the same group, Sarma *et al.* (8) present an integrated study of mechanical and subcellular function in canine hearts following 29 months of chronic ethanol administration. Utilizing force–velocity curves from glycerinated muscle fibers they present clear-cut evidence of a shift of the curve down and to the left indicating a depression of contractility. On a subcellular basis, they report only a small, but insignificant, decrease in calcium uptake by the sarcoplasmic reticulum from time-matched controls. They conclude, on the basis of this data, that a primary abnormality in the

ethanol depressed myocardium may reside at the level of the contractile proteins. Our data supports their conclusion.

The Triton X-100 methodology represents a significant improvement in studying contractile protein function, for this method eliminates contaminant phospholipid membranes (sarcolemma, sarcoplasmic reticulum, and mitochondria) and permits the study of "purified" myofibrils (15). This methodology has been applied in the study of contractile protein dysfunction due to acidosis (24), cardiogenic endotoxin shock (22), and in ischemia/reperfusion injury (25). In a recent report (25) Hess *et al.* demonstrated that in normothermic ischemic arrest for 20 min, subendocardial myofibrillar ATPase function was depressed and that this subcellular dysfunction preceded measurable mechanical dysfunction. This seems to be the specific abnormality identified in this study. The presence of normal mechanical performance in rats after as much as 17 weeks of ethanol ingestion indicates that no significant cardiomyopathy has occurred within the period.

The mechanism by which ethanol or its circulating metabolite, acetaldehyde, depresses contractile function is most probably complex and continues to be an active field of investigation. A direct example of the complexity of the system is the demonstration that acetaldehyde depresses microsomal protein synthesis from cardiac muscle (26) and this in turn could partially be responsible for the depression of the sarcoplasmic reticulum observed in the latter stages of alcoholic cardiomyopathy (8, 12).

Similarly, both acetaldehyde and ethanol are capable of altering contractile protein function. In ethanol-induced skeletal muscle myopathy, Rubin and co-workers (27) have demonstrated that actomyosin ATPase activity and calcium sensitivity are decreased in baboons and human volunteers. Further insight into this contractile protein defect is provided by Puszkin and Rubin (28) when they demonstrated that *in vitro,* ethanol and acetaldehyde did not depress skeletal muscle adenosine triphosphatase activity but inhibited the association of actin and myosin (29) probably by inhibiting the binding of calcium to troponin (12). If it is valid to argue from skeletal muscle data to cardiac muscle, by combining our observations of a decrease in myofibrillar ATPase activity (activation) and the observation of Puszkin and Rubin (28, 29) one could speculate that the specific defect in contractile protein function in alcoholic cardiomyopathy is an alteration of troponin-C–calcium binding interaction. This present study is the first to demonstrate a slow depression of cardiac myofibrillar ATPase activity during chronic ethanol administration and suggests that the myofibril may be an early site at which ethanol or acetaldehyde causes injury.

1. Burch, G. E., and Giles, T. D., Amer. J. Med. 141, Editorial (1971).
2. Rettig, G., Gibson, K., and Harris, P., J. Mol. Cell. Cardiol. 11, 127 (1979).
3. Bing, R. J., Tillmanns, H., Fauvel, J., Seeler, K., and Mao, J. C., Circ. Res. 35, 33 (1974).
4. Burch, G. E., Colcolough, H. L., Harb, J. M., and Tsui, C. Y., Amer. J. Cardiol. 27, 522 (1971).
5. Hirota, Y., Bing, O. H. L., and Ablemann, W. H., J. Mol. Cell. Cardiol. 8, 727 (1976).
6. Maines, J. E., III, and Aldinger, E. E., Amer. Heart J. 73, 55 (1967).
7. Pachinger, O. M., Tillmanns, H., Mao, J. C., Fauvel, J., and Bing, R. J., J. Clin. Invest. 52, 2690 (1973).
8. Sarma, J. S. M., Ikeda, S., Fischer, R., Maruyama, Y., Whieshaar, R., and Bing, R. J., J. Mol. Cardiol. 8, 951 (1976).
9. Williams, E. S., and Li, T. K., J. Mol. Cell. Cardiol. 9, 1003 (1977).
10. Alexander, C. S., Forsyth, G. W., Nagasawa, H. T., and Kohloff, J. G., J. Mol. Cell. Cardiol. 9, 235 (1977).
11. Alexander, C. S., Sekhri, K. K., and Nagasawa, H. T., J. Mol. Cell. Cardiol. 9, 247 (1977).
12. Rubin, E., New Eng. J. Med. 301, 28 (1979).
13. Regan, T. J., Kahan, M. I., Ettinger, P. O., Haider, B., Lyons, M. M., Oldewurtel, H. A., J. Clin. Invest. 54, 740 (1974).
14. Alexander, C. S., Brit. Heart J. 29, 200 (1967).
15. Solaro, J., Pang, D. C., and Briggs, F. N., Biochim. Biophys. Acta 245, 259 (1971).
15. Solaro, J., Pang, D. C., and Briggs, F. N., Biochim. Biophys. Acta 245, 269 (1971).
16. Scheuer, J., and Bhan, A. K., Circ. Res. 45, 1 (1979).
17. Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. 193, 265 (1951).

18. Fanburg, B., and Gergeby, J., J. Biol. Chem. **240**, 2721 (1965).
19. Mason, D. T., Spann, J. F., and Zelis, R., Amer. J. Cardiol. **26**, 248 (1970).
20. Hess, M. L., Circ. Shock **6**, 119 (1979).
21. Solaro, R. J., and Briggs, F. N., Circ. Res. **34**, 531 (1974).
22. Bruni, F. D., Soulsby, M. E., and Hess, M. L., Amer. J. Physiol. **235**, H150 (1978).
23. Gimeno, A. L., Gimeno, M. F., and Webb, J. L., Amer. J. Physiol. **203**, 194 (1962).
24. Kentish, J. C., and Naylor, W. G., J. Mol. Cell. Cardiol. **11**, 611 (1979).
25. Hess, M. L., Barnhart, G. R., Crute, S., Komwatana, P., Krause, S., and Greenfield, L., J. Surg. Res. **26**, 175 (1979).
26. Schreiber, S. S., Oratz, M., and Rothschild, M. A., J. Mol. Cell. Cardiol. **6**, 207 (1974).
27. Rubin, E., Katz, A. M., Lieber, C. S., Stein, E. P., and Puszkin, S., Amer. J. Pathol. **83**, 499 (1976).
28. Puszkin, S., and Rubin, E., Science **188**, 1319 (1975).
29. Puszkin, S., and Rubin, E., Arch. Biochem. Biophys. **177**, 574 (1976).

Received January 29, 1980. P.S.E.B.M. 1980, Vol. 164.