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Cardiac Energy Stores and Creatine in Experimental Cardiac Hypertrophy (40519)

PATRICIA J. REILLY AND JON D. COOKSEY

Marshall University, School of Medicine, Huntington, West Virginia 25701

A major postembryonic adaptation of the heart is the ability of this organ to respond to various stimuli by increasing in mass. All of these stimuli, clinical and experimental, increase the tension or stress in the heart by dilating it or increasing the pressure developed by the heart. According to a modification of Laplace's law relating to stress $\sigma = P \cdot r/h$ the stress (σ) in the wall of a sphere is dependent upon the pressure (P) within the wall, the radius (r) of the sphere, and the thickness (h) of the wall of the sphere. Although the heart is ellipsoidal, this law is applicable (1). When the stress in the heart wall is increased by either a pressure or volume overload, the heart adapts to return the stress on the myocardial fibers toward normal. It does so by hypertrophying and thus increasing wall thickness.

The biochemical mechanism by which increased heart tension results in cardiac hypertrophy is unknown. This mechanical–biochemical coupling may occur at one or several intracellular sites. The existing hypotheses concerning the stimulus to hypertrophy in the heart may be summarized as follows: (a) Increased work demand on the heart may lead to local tissue hypoxia or depletion of energy stores, with accumulation of materials causing genetic activation; (b) increased wall tension or hypoxia may lead to macromolecular breakdown and a similar accumulation of control molecules; (c) stretching of the muscle cells secondary to an increased volume or pressure overload may activate growth processes, perhaps by altering cell membrane properties; (d) humoral or hormonal factors may be involved (2).

Recent studies using monolayer cultures of skeletal and myocardial fetal muscle cells have shown that creatine, an end product of muscular activity, stimulates contractile protein synthesis and therefore may be the chemical signal coupling increased musclar activity and increased muscle mass (3). In the present study we have serially measured the myocardial concentration of adenosine triphosphate (ATP), creatine phosphate (CP) and creatine (Cr) during the development of cardiac hypertrophy induced by constriction of the ascending aorta in young rats.

Materials and methods. Female Wistar rats weighing 230–260 g were used in the study. Previous studies have shown that smaller, younger rats develop a greater degree of hypertrophy and do so at a faster rate than larger, older animals (4). The animals were housed in individual cages in the animal care facility and received water and a standard laboratory diet (Purina Rat Chow) *ad libitum*.

The animals were divided into eight groups, each consisting of 14–20 animals. A control group was used to establish normal values. The remainder of the animals underwent aortic constriction (banding) as described later. Groups of rats were sacrificed at 6 hr, 24 hr, 48 hr, 72 hr, 7 days, 10 days and 14 days after constriction of the aorta. A sufficient number of animals (14–20) were banded for each time interval to allow for the simultaneous sacrifice of animals for both chemical analyses and heart weight. This allowed comparison of the biochemical changes with the degree of hypertrophy developed at that time interval after aortic constriction. A sham-operated group underwent the same surgical procedure as the aortic banded groups, except for the placement of a metal clip around the aorta. This group was sacrificed at 72 hr postbanding for ATP, CP and Cr analyses and at 7 days postbanding for heart weight because aortic constriction produced the largest deviation from control values at these time intervals.

The procedure for aortic constriction was carried out under intraperitoneally administered sodium methohexital (100 mg/kg). Animals were manually ventilated using a polyethylene tube inserted into the pharnyx. A gas mixture of 95% oxygen and 5% carbon dioxide was used for ventilation. The right chest was opened and the ascending aorta

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was dissected free and lifted to allow placement of a tantalum clip around it. The size of the clip was standardized by adjusting a set screw in the clip holder by placing it around a 0.63 mm wire. In a preliminary study it was determined that this size clip would produce cardiac hypertrophy in rats with a mortality less than 10%. The chest was closed with suture and metal clips.

The degree of hypertrophy produced by aortic constriction was estimated by the ratio of heart weight to body weight (HW/BW). Animals that were sacrificed for HW/BW were weighed immediately before removal of the heart. After careful trimming of excess tissue, the ventricles were cut open to allow thorough rising of blood from the heart in physiological saline. The hearts were blotted and weighed on a Mettler P136 balance. Water content was measured by drying a 100 ± 15 mg sample of the left ventricle in a preweighed, dried crucible. After 10 days in a 70° oven, the dry weight was recorded at room temperature on two consecutive days to assure stability of the weight.

Collection of tissue samples for ATP and CP analyses was carried out as follows. Animals were anesthetized with sodium methohexital and ventilated with the O_2–CO_2 gas mixture for 30 seconds before opening the chest. In a preliminary study blood was aspirated from the left ventricle while the animal was ventilated and analyzed for O_2, CO_2 and acid-base status. Blood was 99% saturated with O_2 (pO_2 = 180–400 mm Hg) and had a pH of 7.40–7.55. These precautions were taken to eliminate hypoxia as a possible factor in the change of myocardial ATP and CP content, which has been previously reported (5). While the animal was ventilated, the chest was opened and the right ventricle was held with forceps while the left ventricle was clamp-frozen *in situ* with tongs made of light metal blocks which had been precooled in liquid N_2. Portions of the sample projecting over the edge of the blocks were broken off and the remaining sample was stored at −90°.

Utilizing a mortar and pestle in a cold room at −20°, the frozen portion of the left ventricle was pulverized to a fine powder under a layer of liquid nitrogen. A 100 ± 15 mgm sample was placed in a Roller-Smith torsion balance, the weight recorded and the sample was transferred to a tube containing 4 vol of 3 *M* perchloric acid. The tube was mixed briefly to wet the tissue and frozen immediately in liquid n_2.

This sample was homogenized for 30 seconds with a motor driven, matched ground glass pestle in an ice-water bath and diluted with 4 vol of water before centrifugation at 4000*g* for 10 min at 4°. The deproteinized perchloric acid extract was neutralized with a solution of 2 *N* KOH, 0.4 *M* imidazole and 0.4 *M* KCl. The extract was stored at −90° until the day of assay.

ATP was determined spectrophotometrically by the enzymatic method of Lamprecht and Trautschold (6). CP was determined in the same reaction mixture following the addition of adenosine diphosphate (ADP) and creatine kinase (CK) to a final concentration of 0.33 m*M* and 0.04 mg/cm^3 respectively (7). The amount of ATP and CP was directly proportional to the amount of NADPH produced in the respective reactions. To check the activity of enzymes used in this assay, ATP and CP standards were run with each assay as well as duplicate samples of different volumes. Extracts from control hearts were run in the same assay as extracts from aortic banded hearts to minimize inter-assay variability.

Free creatine was determined by the spectrophotometric measurement of the color that develops when creatine reacts with diacetyl and L-napthol (8). This method allows the determination in an alkaline medium in which creatine is stable.

The data is expressed as mean values ± SE of the mean (SEM). The animals in each group were compared with animals in the control group using a student's t-test. The 0.05 confidence was considered significant.

Results. Aortic constriction resulted in a mean 53% increase in the heart weight to body weight ratio (HW/BW) within 7 days ($p < 0.001$; Table I). The hearts were significantly hypertrophied (8%) by 24 hours following aortic banding ($p < 0.05$) and continued to increase in weight until 7 days, after which there was no further change in the 14-day period. There was no significant difference in HW/BW between control and sham-operated groups ($p < 0.83$).

Left ventricular ATP concentration was

significantly reduced at 6 hr following aortic banding ($p < 0.001$) and continued to decrease for 24 hr post-banding ($p < 0.001$) to a level that was 30% below the control value (Table II). The concentration of ATP remained at this low level at 72 hr. At 7 days after banding, the ATP concentration had increased from the low values found in the first 3 days, but remained at a level that was 20% below the control value at 7, 10 and 14 days.

Due to the development of cardiac hypertrophy, the absolute amount of ATP per heart was actually greater at 7 days than the absolute amount in control hearts, and continued to increase from day 7 to day 14. The absolute amount of ATP per heart was calculated indirectly by multiplying the left ventricular concentration of ATP in moles/g times the whole heart weight in grams. Although the whole heart weight also includes the weight of the right ventricle and atria, these weights remain essentially constant during the development of left ventricular hypertrophy produced by constriction of the aorta. The increase in heart weight is almost entirely due to an increase in the weight of the left ventricle. The concentration of ATP and CP are lower in the right ventricle so this calculation is not intended as an accurate value for absolute amount of ATP and CP per heart, but rather as a means of adjusting the value of ATP and CP concentration to the increase in heart weight (left ventricular weight) produced by aortic constriction (9).

There was no significant difference in the concentration of ATP between control and sham-operated groups ($p < 0.53$; Table II).

Left ventricular CP concentration was not significantly reduced at 6 hr postbanding ($p < 0.17$), but was significantly lower at 24 hr postbanding ($p < 0.002$) and continued to

TABLE I. HEART WEIGHT, BODY WEIGHT, HEART WEIGHT TO BODY WEIGHT RATIO AND LEFT VENTRICULAR WATER CONTENT FOLLOWING AORTIC CONSTRICTION.[a]

Group	Heart Weight (wet, mg)	Body weight (g)	Heart weight (mg) / body weight (g)	Percentage water
Control (13)	908 ± 37	253.4 ± 5.5	3.57 ± 0.11	76.7 ± 0.2
Sham (8)	940 ± 22	263.0 ± 7.2	3.61 ± 0.07	77.2 ± 0.2
6 hr (9)	871 ± 46	258.6 ± 3.9	3.47 ± 0.09	76.1 ± 0.7
24 hr (11)	928 ± 24	240.6 ± 5.0	3.86 ± 0.08**	77.5 ± 0.5
48 hr(6)	1019 ± 39	239.7 ± 2.1	4.25 ± 0.17**	78.0 ± 0.4**
72 hr (10)	1042 ± 38**	238.7 ± 2.7	4.42 ± 0.21*	77.7 ± 0.3**
7 days (10)	1297 ± 55*	244.6 ± 2.3	5.46 ± 0.30*	78.1 ± 0.2*
10 days (7)	1412 ± 54*	251.8 ± 1.9	5.62 ± 0.23*	78.1 ± 0.2*
14 days (5)	1435 ± 81*	256.3 ± 7.9	5.64 ± 0.43*	77.8 ± 0.4**

[a] Data is expressed as mean ± SEM. Numbers in parentheses represent the number of animals in the group.
* $p < 0.001$.
** $p < 0.05$.

TABLE II. ATP, CP AND CREATINE IN THE LEFT VENTRICLE AFTER AORTIC CONSTRICTION.[a]

	ATP		CP		Creatine	
Group	*μmoles* g (wet)	Absolute Amount	*μmoles* g (wet)	Absolute amount	*μmoles* g (wet)	Absolute amount
Control (11)	5.39 ± 0.06	4.89	6.63 ± 0.16	6.02	18.15 ± 0.79	16.48
Sham (8)	5.35 ± 0.10	5.03	6.26 ± 0.29	5.88	18.51 ± 0.75	17.40
6 hr (10)	4.63 ± 0.20*	4.03	6.18 ± 0.28**	5.38	14.93 ± 0.80**	13.00
24 hr (10)	3.82 ± 0.11*	3.54	5.65 ± 0.34*	5.24	10.20 ± 0.77*	9.47
48 hr (8)	3.89 ± 0.16*	3.96	5.15 ± 0.30*	5.25	11.11 ± 1.07*	11.32
72 hr (8)	3.93 ± 0.19*	4.10	4.60 ± 0.34	4.79	9.94 ± 1.23*	10.36
7 days (9)	4.23 ± 0.12*	5.49	3.87 ± 0.35	5.02	13.22 ± 0.59	17.15
10 days (6)	4.37 ± 0.11*	6.17	4.94 ± 0.23*	6.98	13.92 ± 1.14**	19.66
14 days (8)	4.45 ± 0.14*	6.39	4.72 ± 0.40	6.77	14.36 ± 0.91**	20.61

[a] Note: Data is expressed as mean ± SEM. Numbers in parentheses represent the number of animals in the group.
* $p < 0.01$.
** $p < 0.05$.

decrease until 7 days after banding to a level that was 40% below the control value ($p < 0.001$; Table II). The concentration of CP showed a small increase from day 7 to day 10, but did not change significantly from 10 days to 14 days. It remained at a level that was 30% below the control value ($p < 0.001$). Again the absolute amount of CP per heart (calculated) was greater at 10 and 14 days than the absolute amount in control hearts. There was no significant difference in CP concentration between control and sham-operated groups ($p < 0.25$; Table II).

The concentration of creatine in the left ventricle was significantly decreased at 6 hours after aortic constriction ($p < 0.01$) and continued to decrease to a level at 72 hr that was almost half of the control value ($p < 0.001$; Table II). The low level of creatine found at 72 hr had increased at 7 days ($p < 0.001$; Fig. 1) and continued to increase slightly at 10 and 14 days. Although the concentration of creatine at 14 days was still 20% below the control values, the absolute amount of creatine per heart (calculated) was greater than the absolute amount in control hearts. There was no significant difference in the creatine concentration in the hearts of control and sham-operated animals ($p < 0.75$).

There was a small (1%) but significant increase in left ventricular water content in the hypertrophied hearts ($p < 0.01$; Table I). However, this difference is so small it cannot account for the large decreases observed in ATP, CP and Cr concentrations or the large increases (53%) in the weight of the heart observed following aortic constriction. There was no significant difference in the water content of control and sham-operated groups ($p < 0.16$).

Eighteen of the animals died spontaneously following aortic banding secondary to congestive heart failure. Congested lungs, fluid in the chest and respiratory tract, "nutmeg" liver (congested) and dilated heart were observed when the animals were examined post mortem.

Discussion. Hypertrophy is recognized as a fundamental adaptation of the heart to a variety of stresses. However, the frequent deterioration of initially compensatory hypertrophy into congestive heart failure, even without increasing stress on the heart, implies that the hypertrophied heart may be abnormal, even though it has adapted to the stress. Studies on oxygen consumption and contractility show the energetics of pressure–hypertrophied rat right ventricle to be abnormal. The mechanism suggested for the paradoxical increase in oxygen consumption is an increase in the nonphosphorylating state four mitochondrial respiration (10). The regulation of intracellular calcium depends on an ATP-dependent transport process by the sarcoplasmic reticulum (11). Defects have been demonstrated in the sarcoplasmic reticulum's ability to bind and release calcium in both

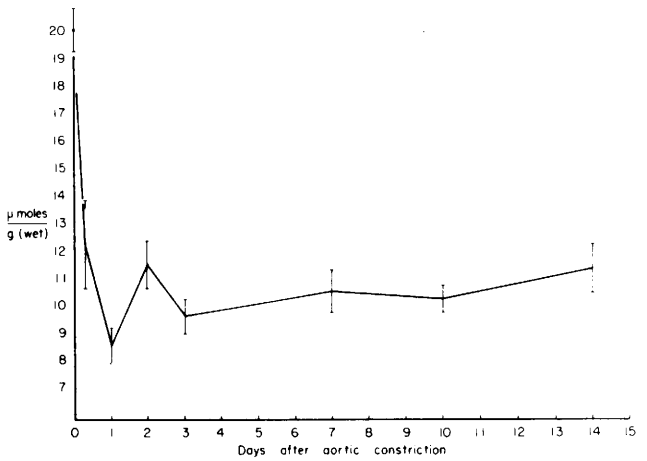

FIG. 1. Time course of the changes in the concentration of creatine in the left ventricle following aortic constriction. Values are expressed as mean ± SEM.

human and animal heart muscle showing diminished contractility (12). This defect in the calcium sequestering ability of the sarcoplasmic reticulum may result in increased uptake of calcium by mitochondria to keep the intracellular calcium concentration normal. This calcium uptake would use ATP or the energy high state generated by electron transport which might have been used to make ATP. Mitochondria may function as calcium segregating organelles in the heart, possibly by supplementing the activity of the impaired sarcoplasmic reticulum (13). Mitochondria can accumulate large amounts of calcium either in the matrix or bound to the membrane. *In vitro* mitochondria prefer to use electron transport energy to take up calcium than to phosphorylate ADP. This might provide some explanation for the failure of the ADP, produced in the utilization of ATP, to stimulate mitochondrial oxidative phosphorylation in hypertrophied heart muscle and hence for the lower levels of ATP and CP found in the hearts of animals with stable hypertrophy (7, 10 and 14 days) in the present study.

The results of this study agree with the explanation suggested by Rabinowitz and Zak: during the rapidly developing stages of hypertrophy, the phosphate potential decreases, but after the accumulation of mitochondria, there is a partial normalization of energy stores (9). However, they also pointed out that even though the ATP and CP levels correlate with the stimulation of hypertrophy, we still do not know how the stimulation is mediated.

Ingwall has proposed that creatine is the direct chemical signal coupling increased activity to increased contractile protein synthesis in hypertrophy (3). She has demonstrated that muscle-specific protein synthesis is selectively stimulated by creatine in culture skeletal muscle cells and in the fetal mouse heart in organ culture. However, it has not been demonstrated that creatine functions this way *in vivo*. In an attempt to test whether creatine functions as a stimulus to muscle protein synthesis in hypertrophy, the time course of the changes in creatine concentration was determined in the present investigation. The results of this study clearly show a marked decrease in the concentration of creatine six hours after aortic constriction and the time course of creatine follows the same direction as that of CP. This finding was contrary to what was expected initially. Since creatine is a breakdown product of CP during muscular activity, it was expected than an accumulation of creatine would be observed as the level of CP decreased. However, the creatine liberated in the transfer of the high energy phosphate from CP to ADP is readily permeable to the muscle cell membrane and does not accumulate in the cell (14). *In vitro* studies on the role of the creatine-CP system in muscle have demonstrated increased intracellular creatine in the present of creatine added to the culture medium (15). Furthermore, substantial increases in the intracellular concentration of CP were also observed in the same study. The level of CP was directly proportional to the amount of creatine in the medium. This observation led to the suggestion that creatine serves as a regulatory substance in the energetics of the muscle cell rather than simply as an energy shuttle. According to this theory, the results of the present study would be interpreted in a different way: the creatine which leaked out of the cell resulting in lower intracellular concentration of creatine caused a decrease in intracellular high energy phosphate stores proportional to the decrease in creatine. According to Seraydarian, creatine is a much better candidate than ADP to serve as the effector molecule which stimulates oxidative phosphorylation in the mitochondria.

Although the proposed models of the role of creatine work well *in vitro*, it does not appear that creatine serves as the chemical stimulus which initiates the development of cardiac hypertrophy in the intact animal. In the present study, the concentration of creatine decreased as the hypertrophy developed. Other *in vivo* studies have also shown decreased creatine concentration in right ventricular hypertrophy of heart failure (5, 16). It should be pointed out that heart muscle cell cultures have no neural or hormonal controls, and the diffusion barrier is minimal in a monolayer cell culture or organ culture. In addition, there is no stress on cultured heart cells, while in the living animal, there are continuous pressure changes in the heart, especially during developing hypertrophy. Because of these important differences it is

difficult to extrapolate from these studies to *in vivo* functions.

Summary. The biochemical mechanism by which increased heart tension results in cardiac hypertrophy is unknown. *In vitro* studies using muscle cell cultures have suggested that the concentration of creatine is the chemical mediator of this hypertrophy. To examine this mechanism *in vivo*, the myocardial concentration of ATP, CP and Cr was serially measured following aortic constriction. The aortic constriction resulted in a 53% increase in heart weight after 2 weeks. The concentration of ATP, CP or Cr decreased following aortic constriction to a level 20–50% below control values. These data suggest that the creatine concentration *in vivo* is not the stimulus for hypertrophy, but the decreased creatine concentration in hypertrophied hearts may limit the level of ATP and CP that can be maintained during the development of cardiac hypertrophy.

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