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LACTATE DEHYDROGENASE AND PYRUVATE KINASE IN RAT HEART DURING SIDEROPENIC ANEMIA

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SUMMARY

Lactate dehydrogenase isozyme pattern and activity and pyruvate kinase activity were studied in animals with severe sideropenic anemia. This condition was induced by feeding rats a diet of non-fat dry milk–sucrose from the time of weaning. Anemia was indicated by the lowering of blood hemoglobin levels to 25 and 20% of normal in two groups after 30 and 85 days, respectively. In both anemic groups there was accompanying cardiac enlargement and retardation of body weight gain. Statistically significant increases in cardiac lactate dehydrogenase (25–33%) and pyruvate kinase (25%) activity were observed in both groups relative to controls. An alteration of the normal myocardial lactate dehydrogenase isozyme pattern in the direction of increased lactate dehydrogenase-5 took place in both groups of anemic rats. This resulted from a dramatic increase in the M-lactate dehydrogenase subunit contribution (45–54%) to total lactate dehydrogenase activity.

INTRODUCTION

Animal studies show that chronic severe anemia results in cardiac enlargement [1, 2]. However, possible alterations of glycolytic enzyme activity of such hearts has received little attention. It is known, for example, that in the anemic condition cardiac output and energy expenditure of the heart are elevated several-fold [3]. Yet, studies with hearts of anemic animals suggest that capillary density is no greater than in normal hearts [4]. This coupled with the resulting higher oxygen demands of the heart muscle and reduced ability of the anemic animal's circulatory system to transport oxygen may lead to decreased oxygen tension in the myocardium. It is conceivable that such a condition could force increased reliance on glycolysis. Experimentally an increased capacity for anaerobic glycolysis is suggested by studies demonstrating that both the isolated right ventricle and the heart-lung preparation of anemic rats show a higher tolerance to anoxia than hearts from normal animals [4, 5]. Nevertheless, evidence that changes in glycolytic enzyme activity occur in hearts of such anemic animals has been lacking.

The present investigation was carried-out in an effort to assess the effect of anemia on two glycolytic enzymes in rat heart; lactate dehydrogenase (L(+)-lactate:

NAD^+ oxidoreductase, EC 1.1.1.27) and pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40).

MATERIALS AND METHODS

Induction of anemia

Male rats of a Charles River CD strain were used (Charles River Breeding Laboratories, Wilmington, Mass., U.S.A.). Newly weaned (21-day-old) animals were transferred to individual cages and given ad libitum a diet of either 75% non-fat dry milk (Carnation Co., Los Angeles, Calif., U.S.A.)–25% sucrose similar to that of Korecky et al.[6] or Purina rat chow. Animals maintained on the milk–sucrose diet were provided with double distilled water from all glass drinking tubes. Controls drank tap water from standard stainless steel drinking tubes. Two groups of rats were made anemic by feeding the milk–sucrose diet over periods of 30 days (beginning body wt 57.0 $\pm$ 3.0 g) and 85 days (beginning body wt 46.0 $\pm$ 2.0 g). Blood hemoglobin concentration was monitored[7] periodically during the course of anemia induction and at the time the animals were sacrificed.

Tissue preparation

Animals were sacrificed by decaptitation. Hearts were immediately excised, placed in ice cold 0.9% saline and trimmed of fat and major vessels. Hearts were frozen at solid CO_2 temperature, weighed to the nearest mg and pulverized in the cold to a fine powder.

Lactate dehydrogenase assay

Portions of the frozen heart powder were homogenized in glycine–NaOH buffer (0.05 M, pH 9.6) and made up to volume with additional buffer. This homogenate was centrifuged at 12 000 $\times$ *g* at 4 °C for 15 min and the supernatant used for electrophoresis. Separation of lactate dehydrogenase isoenzymes was carried out using an E-C vertical acrylamide gel electrophoresis apparatus (E-C Apparatus Co., Phil., Pa., U.S.A.). The heart supernatant samples containing 15% sucrose and 0.005% (w/v) bromophenol blue (tracking dye) were placed into slots (3 mm wide $\times$ 10 mm long) in 7% cyanogum-41 gel made up with 0.05 M glycine–NaOH buffer (pH 9.6). The upper and lower compartments of the electrophoresis apparatus also contained this buffer solution. Lactate dehydrogenase was electrophoresed toward the anode at 300 V for 4 h at 19–21 °C. The electrophoresed gels were developed for 45 min in the dark at 37 °C in a staining solution containing the following components in mM concentration: sodium lactate, 1330; phenazine methosulfate, 0.054; NAD^+, 1.00; nitro blue tetrazolium, 0.33; and Tris–HCl buffer (pH 7.4), 200 [8].

Quantitation of the density of staining of lactate dehydrogenase isozymes was performed on the acetic acid fixed electrophoretic gels. This was accomplished in a linear transport equipped Gilford 2400 recording spectrophotometer at 540 nm using a slit plate of 0.1 mm $\times$ 2.36 mm aperture. Gels were scanned at a rate of 1 cm/min and the resulting data (absorbance) was plotted at a chart speed of 0.5 min/inch on a Honeywell recorder. Recorder calibration was 1.0 absorbance units full scale. The area under each isozyme peak was then determined by triplicate measurements with

a hand-operated planimeter. The total lactate dehydrogenase activity of electrophoresed heart supernatant samples is derived from the sum total area under all five isozyme peaks and is expressed in terms of $inch^2$ of paper divided by the duration of staining. That percentage of the total lactate dehydrogenase activity which is contributed by M subunits was calculated from the area under these peaks by an equation of Thorling and Jensen [9] .We will refer to this parameter as the isozyme composition in M. The M and H subunit activities were computed as the product of total lactate dehydrogenase activity and percentage M or H subunit composition.

The efficacy of the above method of quantitating total lactate dehydrogenase activity has been confirmed by studies in this laboratory in which a standard spectrophotometric assay at 340 nm involving the reduction of pyruvate to lactate resulted in lactate dehydrogenase activity nearly identical to those reported here. However, the present method is preferable since by using it isozyme composition in M and H subunits as well as total lactate dehydrogenase activity are obtainable in one assay procedure.

Pyruvate kinase assay

Pyruvate kinase was assayed spectrophotometrically at 340 nm at 37 °C according to the method of Boyer [10]. Frozen heart powder was homogenized in the assay buffer at 0 °C, centrifuged at 12 000 $\times$ *g* in the cold for 15 min and the resulting supernatant used for assay. The reaction mixture had a total volume of 1 ml and contained the following components in mM concentration: imidazole–HCl buffer (pH 7.0), 200; KCl, 100; $MgCl_2$, 4; phosphoenolpyruvate, 1; NADH, 0.16; and 1.0 I.U./ml of crystalline lactate dehydrogenase. The reaction was initiated by addition of ADP at 4 mM final concentration. Loss of absorbance in blanks lacking ADP was negligible.

Enzyme units

Enzyme activities are expressed per g of heart protein $\pm$ S.E. Protein was assayed [11] using bovine serum albumin as standard.

Statistical analysis

The Student *t* test was used for determination of statistical significance [12]. Differences between means which resulted in probability values (*P*) smaller than 0.05 were considered significant.

Reagents and enzymes

The biochemicals and enzymes used in this study were obtained from the following sources: bovine serum albumin, General Biochemicals, Chagrin Falls, Ohio, U.S.A.; *N,N,N′,N′*-tetramethylethylene diamine, Eastman Kodak, Rochester N.Y., U.S.A.; potassium persulfate and cyanogum 41 (acrylamide), E-C Apparatus Co., Philadelphia, Pa., U.S.A.; LDH, NAD^+, NADH, ADP, phosphoenolpyruvate, nitro blue tetrazolium, phenazine methosulfate, glycine, sodium lactate, bromophenol blue, Sigma Chemical Co., St. Louis, Mo., U.S.A.; KCl, $MgCl_2$ and other inorganic salts, Mallinckrodt Chemical Co., St. Louis, Mo., U.S.A.

RESULTS

Anemia and heart size

The hemoglobin, body weight and wet heart weight of anemic and control animals are presented in Table I. Both the rats fed the milk–sucrose diet for 30 and 85 days showed very low blood hemoglobin concentrations relative to control rats fed standard rat chow (28 and 20% of control, respectively). It may also be noted that the hemoglobin concentration of the 30-day anemic control group (51 days of age at sacrifice) was 2.46 g/100 ml lower than the 85-day anemic control group (106 days of age at sacrifice). This results from the normal rise in hemoglobin concentration in rats from a value of 10.0 g/100 ml at 21 days of age to 15.0 g/100 ml in the adult at

TABLE I

EFFECTS OF FEEDING RATS A NON-FAT DRY MILK–SUCROSE DIET (BEGUN AT 21 DAYS OF AGE) OVER PERIODS OF 30 AND 85 DAYS ON HEMOGLOBIN, BODY WEIGHT AND HEART WEIGHT

Number of animals used are given in parenthesis. Except for % of predicted heart wt, all means given ± S.E. *P* values between means in each column given.

Group	Hemoglobin (g/100 ml)	Body wt (g)	Heart wt		
			Measured (g)	Predicted (g)	% of predicted (g/g × 100)
Control (11)	12.49 ±0.33	269.7 ±7.6	0.922 ±0.030	0.926 ±0.030	99.57
30-day anemic (11)	3.45 ±0.17	137.7 ±6.2	0.800 ±0.090	0.523 ±0.020	152.96
P	<0.001	<0.001	>0.05	<0.01*	
Control (11)	14.95 ±0.18	413.1 ±22.1	1.060 ±0.040	1.050 ±0.050	100.95
85-day anemic (13)	3.05 ±0.15	151.9 ±6.8	1.310 ±0.060	0.555 ±0.020	236.04
P	<0.001	<0.001	<0.01	<0.001*	

* Anemic measured vs anemic predicted.

100 days of age and beyond (Penney, D., unpublished observations). Severe retardation of body wt gain was experienced by both anemic groups relative to their respective controls. The 30- and 85-day anemic control group increased in body wt by 373 and 798%, respectively, while the 30- and 85-day anemic animals increased 142 and 230%, respectively. However, regardless of differences in body wt, it can be seen that the 85-day anemic group had a significantly ($P < 0.01$) larger (23.58%) wet heart wt relative to the control group. The hearts of the 30-day anemic animals were not absolutely heavier than their respective control hearts. In order to correct for the differences in final body wt which occurred so as to make valid comparisons of heart weight in anemic and control rats of similar body wt possible, the following procedure was adopted. Body and wet heart wt data on a large group of normal male rats in the body wt range 100–450 g were obtained. Since it is well known that the wet heart wt

increases almost directly with body wt in the growing semi-adult rat [13], the wet heart wt of the normal rat may be computed directly from body wt using linear regression equations relating wet heart to body wt. In this way it was possible to compare the wet heart wt of anemic rats to wet heart wt (predicted) of identical body wt control animals. A method similar to this has been used previously by Korecky et al. [2] and Grove et al. [14] As can be seen the measured wet heart wt of the 30- and 85-day anemic groups were significantly larger than the predicted wet heart wt of equal body wt controls computed in this manner (153 and 236%, respectively).

Enzyme changes

The lactate dehydrogenase isozyme composition in M of both 30- and 85-day anemic animals increased (6.03 and 5.41%, respectively) relative to controls (Table II). This isozyme shift in both anemic groups was accompanied by a significant increase in total lactate dehydrogenase activity over their respective controls (33.82% and

TABLE II

EFFECTS OF FEEDING RATS A NON-FAT DRY MILK–SUCROSE DIET (BEGUN AT 21 DAYS OF AGE) OVER PERIODS OF 30 AND 85 DAYS ON LACTATE DEHYDROGENASE ISOZYME COMPOSITION IN M, ACTIVITY OF M AND H LACTATE DEHYDROGENASE SUBUNITS, TOTAL LACTATE DEHYDROGENASE ACTIVITY AND PYRUVATE KINASE ACTIVITY OF HEART

Number of animals used are given in parenthesis. Means given ± S.E. *P* values between means in each column given.

Group	Lactate dehydrogenase (% M)	Lactate dehydrogenase activity (inch^2 × 1000 per min/g protein)			Pyruvate kinase activity (μmoles NADH oxidized per min/g protein)
		M subunit	H subunit	Total	
Control	36.02	5.96	10.57	16.53	489.0
(11)	±0.99	±0.51	±0.61	±1.12	±33.0 (7)
30-day anemic	42.05	9.30	12.82	22.12	614.0
(11)	±1.33	±1.03	±1.07	±2.10	±25.0 (7)
P	<0.01	<0.05	>0.05	<0.05	<0.05
Control	32.82	5.33	10.91	16.24	492.0
(10)	±1.37	±0.34	±0.61	±0.95	±13.0 (8)
85 day anemic	38.23	7.73	12.60	20.33	615.0
(13)	±1.15	±0.52	±0.75	±1.27	±16.0 (8)
P	<0.01	<0.01	>0.05	<0.01	<0.001

25.18%, respectively). Moreover, this increase in total lactate dehydrogenase activity in both groups was the result of a significant rise in M subunit (54.04 and 45.03%, respectively). The H subunit contribution to total lactate dehydrogenase activity showed only a small and statistically insignificant increase in both groups. It may also be noted that the isozyme composition in M of the hearts of the younger control rats (51 days at sacrifice) was higher than that of the older control rats (106 days at sacrifice). This effect was reflected in the isozyme composition in M of the two groups of anemic animals as well.

Pyruvate kinase activity of hearts of both 30- and 85-day anemic animals was elevated 25.65 and 25.00%, respectively (Table II).

DISCUSSION

The characteristic response of animals fed an iron-deficient diet over long periods of time is greatly depressed hemoglobin and an increase in heart mass [2]. Both of these responses were obtained in animals given the non-fat dry milk–sucrose mixture in this investigation. As in earlier studies, the anemic rats gained weight far more slowly than the normal controls fed standard rat diet [2].

The objective of this investigation was to determine whether alterations in the activities of the glycolytic enzymes lactate dehydrogenase and pyruvate kinase occur in the heart muscle of animals made anemic in this manner. The results of this study clearly show that significant increases in myocardial lactate dehydrogenase and pyruvate kinase activities accompany severe anemia and cardiac enlargement. In addition, the hearts of these animals show a significant increase in lactate dehydrogenase isozyme composition in M. Both the increase in lactate dehydrogenase activity and change in isozyme composition result primarily from substantial increase in the contribution made by the M subunit.

Lactate dehydrogenase is essential under anoxic conditions in most mammalian tissues for regeneration of the coenzyme NAD^+. It commonly occurs in five molecular forms in mammalian tissues; tetramer isozymes arranged in various ratios of two different subunits (M, skeletal muscle; H, heart). A recent report [15] suggests that the skeletal muscle variety of this enzyme (mainly lactate dehydrogenase-5,4M) is capable of readily converting pyruvate to lactate under a wide range of cellular oxygen tensions, while the heart variety (mainly lactate dehydrogenase-1,4H) rapidly forms an inhibitory complex with pyruvate and NAD^+ and may act only as a dehydrogenase of lactate under normal myocardial conditions. Based on this, it might be suggested that the increased M subunit activity and increased total lactate dehydrogenase activity seen in the present study, represents an adaptive mechanism permitting higher rates of pyruvate reduction to lactate at reduced oxygen tension and in turn higher rates of anaerobic glycolysis due to the coupling attendant upon NAD^+ regeneration in the lactate dehydrogenase reaction. Unfortunately this is probably not the case, since it is known that lactate dehydrogenase displays one of the highest activities among glycolytic enzymes in mammalian heart [16]. Furthermore, Williamson [17] has clearly shown that the anaerobic lactate dehydrogenase catalyzed conversion of pyruvate to lactate is normally not a rate-limiting step in the glycolytic pathway. In a similar way one might suggest that an increase in cardiac pyruvate kinase activity would serve to enhance glycolytic capacity. However, the same studies cited above with regard to lactate dehydrogenase [16, 17], as well as others show that pyruvate kinase also is not normally rate-limiting to glycolytic flux. It therefore remains unclear what metabolic function the changes in lactate dehydrogenase and pyruvate kinase might serve in the anemic heart and what part they may play in the enhanced anoxic tolerance of such hearts [4, 5].

A greater lactate dehydrogenase isozyme composition in M of the younger control and anemic hearts was noted in the present study relative to the older control and anemic hearts. This was probably due to the decreasing isozyme composition in M which has been reported to occur in heart during the first few months of postnatal life [18]. A reversal of this developmental change in lactate dehydrogenase subunit composition resulting in increased M subunit also takes place in hearts of dogs with

right ventricular hypertrophy [19] and in human hearts enlarged in association with coronary artery disease [20]. Sobel et al. [21] report a similar change in lactate dehydrogenase during an early period and later (25–50 days) a return to normal in guinea pigs with hypertrophy induced by either aortic or pulmonary artery constriction. In addition, several studies have shown that the subunit composition of lactate dehydrogenase changes to increased percentage of M in the hearts of altitude-acclimated animals [18, 22]. Thus, it is now quite apparent that a number of cardiovascular stresses, including anemia, lead to shifts in lactate dehydrogenase isozyme composition in heart.

In addition to the present work, two other studies, one using monkey heart cell cultures [23] and another using human lymphocytes [24] have reported increases in total lactate dehydrogenase activity resulting from increased M subunit composition relative to H. In both of the latter cases the alterations in lactate dehydrogenase isozymes observed were induced by declining oxygen tension in the gas mixtures used. However, the present study appears to be the first to report both changes in lactate dehydrogenase isozyme composition and elevations in lactate dehydrogenase activity brought about solely by increasing M subunit in stressed mammalian hearts.

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