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Concentrations of D-lactate and its related metabolic intermediates in liver, blood, and muscle of diabetic and starved rats

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Summary. This is a report investigating the methylglyoxal (MG) bypass in animals, by which D-lactate is produced from triosephosphate via MG. Rats were made diabetic using streptozotocin or starved for 72 h. D-Lactate and various metabolites related to it, such as L-lactate, pyruvate, methylglyoxal, glucose, and inorganic phosphate, were measured in the blood plasma, liver, and skeletal muscle of the rats. Diabetic and starved rats had significantly higher levels of D-lactate in plasma, liver, and skeletal muscle compared with the control group. In contrast, pyruvate levels in plasma, liver, and skeletal muscle was markedly lower than normal in diabetic and starved rats. L-Lactate level lowered markedly in plasma, liver, and skeletal muscle of starved rats and elevated in liver of diabetic rats. Differences between plasma L-lactate level for diabetes and control were not significant. MG level was significantly elevated in plasma and depressed in livers and muscles of starved rats as well as livers of diabetic rats. Hepatic glycerol content was markedly increased in those states. Enzyme activities related to D- and L-lactate, such as pyruvate kinase, phosphofructokinase, aldolase, and glyoxalase I, were measured in the livers of these rats. Pyruvate kinase activity decreased in these states, but other enzyme activities showed no significant changes. D-Lactate was much more excreted than L-lactate in the urine of diabetic and fasted rats compared with normal rats.

Key words: D-lactate – L-lactate – Methylglyoxal – Diabetic rat – Starved rat

Introduction

Neuberg [14] as well as Dakin and Dudley [4] discovered a mixture of glyoxalase I and II from animal tissues and yeast, which catalyzed the conversion of MG

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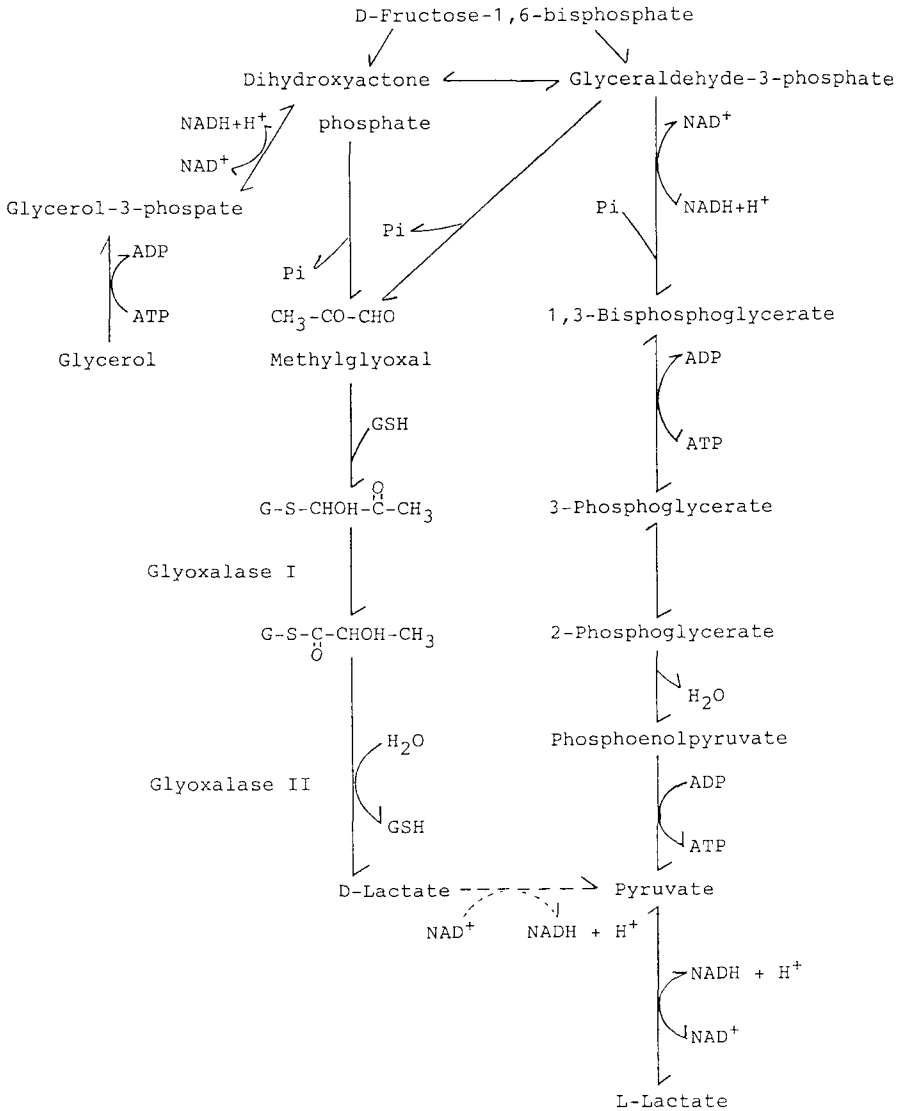

Fig. 1. Pathway for the formation of D-lactate

into D-lactic acid. For about 20 years. MG was thought to be a key intermediate in the catabolism of glucose in animals until Embden and Meyerhof showed that fructose-1,6-bisphosphate was split into two trioses phosphates by muscle extracts [5, 13]; subsequent research concentrated on glycolysis. After about a half century of being disregarded, Cooper and Anderson [3] showed that E. coli contained enzymes that converted dihydroxyacetone phosphate to pyruvate via MG and named this route the MG-bypass (Fig. 1). Although MG, D-lactate, and glyoxalases have been demonstrated to be present in animals, little is known about the physiological significance of the MG-bypass. To solve these problems,

we first devised simple, sensitive, and specific determination methods [16, 17] of MG and D-lactate [15, 19].

In this paper, MG and D-lactate as well as their related metabolites and enzyme activities were examined in diabetic and starved rats using these assay methods.

Materials and methods

Chemicals

Hydrazine sulfate, o-phenylenediamine, streptozotocin, and rabbit muscle aldolase were purchased from Wako Pure Chemicals (Osaka, Japan). Fructose-6-phosphate and γ-glycerophosphate dehydrogenase (γ-GDH) (EC 1.1.1.8) from rabbit muscle, pig heart lactate dehydrogenase (EC 1.1.1.27), phosphoenolpyruvate, and NAD^+ were obtained from Oriental Yeast (Tokyo, Japan). Lithium L- and D-lactate, triosephosphate isomerase (EC 5.3.1.1) from rabbit muscle, and fructose-1,6-diphosphate were from Sigma Chemicals (St. Louis, Mo., USA). DL-6,8-Thioctamide was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). MG was prepared just before use by hydrolysis of the dimethylacetal (Aldrich, Milwaukee, Isc., USA) [10]. D-Lactate dehydrogenase (EC 1.1.1.28) from Staphylococcus sp. and diaphorase (EC 1.6.4.3) from Clostridium kluiveri were kindly supplied by Amano Pharmaceutical (Nagoya, Japan).

Experimental animals

Male Wistar strain albino rats were used for all experiments and fed on standard rat cake MF (Oriental Yeast) or starved for 72 h ad libitum on water. Rats, 120–140 g each, were injected once intraperitoneally with streptozotocin to induce diabetes. Streptozotocin was dissolved in 0.05 *M* sodium citrate (pH 4.5) to make a 5.25% (w/v) solution just before the injection. The control group was injected with physiological saline.

Blood sampling

Rats were injected intraperitoneally with 0.2 ml of 5% pentobarbital. After about 5 min, the abdomens were opened and about 4 ml blood was drawn through cannulation of the abdominal aorta. All needles and syringes were heparinized. Blood samples were centrifuged for 15 min at 1,700 × *g*. The blood sampling was carried out between 7 and 8 a.m.

Tissue preparations

After the blood samples were drawn, the liver and leg skeletal muscles of the rats were immediately excised and rinsed in an ice-cold physiological saline. Livers were perfused with the ice-cold saline and homogenized at 4°C for 30 s in a glass Teflon homogenizer. Muscle was minced with scissors and homogenized at 10,000 rpm for 5 min with a Waring blender in 2 vol. of the ice-cold 10 m*M* potassium phosphate (pH 7.0). The homogenates were centrifuged at 6,000 × *g* at 4°C for 15 min. The supernatant solution was used for analysis.

Determination of metabolite concentrations

D-Lactate and pyruvate were determined by our methods [15, 19], which were based upon the formation of quinoxalinol derivatives. MG was measured as 6,7-dichloro-2-methylquinoxaline by our GLC-method with electron capture detection [16]. L-Lactate concentration was measured colorimetrically [1] or by the UV method (F-kit, Boehringer, Mannheim, FRG) when a large enough quantity of sample had to be assayed. Inorganic phosphate was determined by the colorimetric method of Lanzetta et al. [12]. Measurements of glycerol were performed using an UV detection kit (Boehringer).

Enzyme assays

Activities of 6-phosphofrutokinase (EC 2.7.1.11), pyruvate kinase (EC 2.7.1.40), fructose-bisphosphate aldolase (EC 4.1.2.13), and glyoxalase I (EC 4.4.1.5) were determined by methods previously described in the literature [6, 11, 20, 22], respectively.

Results

Diabetic rats

Body weight of rats after injection of streptozotocin increased more slowly than that of the control group (Table 1). Plasma glucose levels were about 3.8 times higher than that of control rats 17 and 30 days after the injection (Table 1). It is very interesting to note that for each time point the D-lactate content in plasma, liver, and skeletal muscle were markedly elevated in comparison to each control group (Tables 1–3) whereas L-lactate contents did not change in plasma (Table 1), increased in liver (Table 2), and steadily decreased in muscle (Table 3). Another biochemical feature of streptozotocin-injected rats was the marked decrease of pyruvate with time in plasma, liver, and muscle after injection for 17 and 30 days (Tables 1–3). MG level in blood plasma elevated on the third day and showed a tendency to decrease after that (Table 1). The MG contents in liver decreased on the 17th day, but there was no difference in them compared with the control on other days (Table 2). As for MG in skeletal muscle, there was no statistically significant difference between the diabetic and control groups (Table 3). Inorganic phosphate (Pi) contents in plasma and liver were significantly elevated (Tables 1 and 2). Glycerol level in the liver was elevated on the 30th day after injection of streptozotocin (Table 2). Table 4 shows the changes in hepatic enzyme activities in diabetic rats, in which pyruvate kinase activity was lower than that of the control group ($P < 0.001$ on the 17th day and $P < 0.05$ on the 30th day). Other enzymic activities did not change. In Table 5, urinary excretion of L- and D-lactate and the L-lactate/D-lactate ratio are listed for diabetic, starved, and normal rats; diabetic rats excrete much more D-lactate than L-lactate compared with normal group.

Starved rats

As noted in Table 1, starvation for 3 days produced a 20% decrease in body weight and a 15% decrease in blood glucose compared with the control values. As shown in Tables 1–3, the changes of the levels of D-lactate, pyruvate, and Pi in plasma, liver, and muscle of starved rats were the same as those of diabetic rats, i.e., D-lactate and Pi levels increased and pyruvate level decreased compared with the control values, while L-lactate levels in plasma and liver markedly lowered and MG in plasma became elevated. It appears to be a correlation between the concentrations of L-lactate (Y) [nmol/ mg] and pyruvate (X) [nmol/ mg] in muscle after 72-h starvation; $y = 0.415\ x + 12.568$ ($r = 0.925$, $n = 5$). However,the correlation was not observed in plasma and liver: there was no correlation between those of D-lactate and pyruvate in plasma and the tissues. The hepatic content of glycerol was markedly increased after 72-h starvation. Pyruvate kinase activity in the liver of starved rats was significantly decreased ($P < 0.05$), similar to the activity of diabetic rats, whereas the activities of phosphofrutokinase, aldolase, and glyoxalase I were not altered.

Table 1. Plasma levels of D-lactate and its related metabolites as well as body weight of normal, diabetic, and starved rats

Treatment of rat	D-Lactate (μM)	L-Lactate (mM)	Pyruvate (μM)	Methylglyoxal (μM)	Glucose (mg/dl)	Weight (g)	Inorganic Pi (mM)
C (3 days)	99 ± 2.1	0.64 ± 0.91	88.4 ± 10.0	2.42 ± 0.63	138 ± 5.1	150 ± 10.0	5.27 ± 0.78
D (3 days)	122 ± 13.9	0.59 ± 0.06	64.2 ± 6.9	4.73 ± 1.60	293 ± 21.0***	152 ± 11.0***	8.58 ± 0.47*
C (17 days)	110 ± 5.2	0.54 ± 0.07	93.9 ± 7.8	2.19 ± 0.51	150 ± 10.2	226 ± 10.0	4.92 ± 0.41
D (17 days)	192 ± 28.5*	0.54 ± 0.12	58.5 ± 11.7**	1.32 ± 0.40	580 ± 30.0***	206 ± 15.0	6.28 ± 0.65*
C (30 days)	113 ± 11.8	0.57 ± 0.15	99.7 ± 14.2	1.98 ± 0.82	142 ± 7.2	301 ± 20.0	5.58 ± 0.65
D (30 days)	171 ± 24.8*	0.60 ± 0.14	25.9 ± 3.6**	0.94 ± 0.41	540 ± 17.1***	254 ± 20.0	11.0 ± 1.29**
C (72 h)	99 ± 15.1	0.43 ± 0.04	91.0 ± 18.6	1.56 ± 0.23	153 ± 17.2	156 ± 1.8	5.16 ± 0.60
S (72 h)	134 ± 19.8*	0.23 ± 0.01*	36.4 ± 13.6**	3.97 ± 1.43*	130 ± 11.2	126 ± 29.0	11.0 ± 1.41**

C = Control; D = Diabetic; S = Starved
Values are means ± SEM for 5 rats
* $P<0.05$; ** $P<0.01$; *** $P<0.001$

Table 2. Concentrations of D-lactate and its related metabolites in the liver of normal, diabetic, and starved rats

Treatment of rat	D-Lactate (nmol)	L-Lactate (nmol)	Pyruvate (nmol)	Methylglyoxal (nmol)	Inorganic Pi (μmol)	Glycerol (nmol)
C (3 days)	1.01 ± 0.19	6.86 ± 0.94	0.58 ± 0.08	0.09 ± 0.01		
D (3 days)	1.22 ± 0.16	7.87 ± 1.58	0.47 ± 0.10	0.08 ± 0.01		
C (17 days)	1.10 ± 0.11	6.20 ± 0.87	0.57 ± 0.02	0.12 ± 0.01		
D (17 days)	2.76 ± 0.28***	9.61 ± 0.98**	0.14 ± 0.02***	0.06 ± 0.002**		
C (30 days)	1.00 ± 0.08	6.23 ± 1.30	0.62 ± 0.04	0.08 ± 0.003	6.7 ± 0.8	0.02 ± 0.01
D (30 days)	2.34 ± 0.28***	9.52 ± 1.30	0.45 ± 0.10*	0.07 ± 0.004	31.6 ± 1.9***	0.04 ± 0.01*
C (72 h)	1.15 ± 0.7	6.93 ± 0.39	0.58 ± 0.10	0.11 ± 0.004	6.0 ± 0.5	0.02 ± 0.01
S (72 h)	2.34 ± 0.21**	3.66 ± 0.09**	0.31 ± 0.04*	0.06 ± 0.002**	63.2 ± 1.2***	0.06 ± 0.02**

C = Control; D = Diabetic; S = Starved
All values are expressed per mg protein and means ± SEM for 5 rats
* $P<0.05$; ** $P<0.01$; *** $P<0.001$

Table 3. Concentrations of D-lactate and related metabolites in the skeletal muscle of normal, diabetic, and starved rats

Treatment of rat	D-Lactate (nmol)	L-Lactate (nmol)	Pyruvate (nmol)	Methylglyoxal (nmol)
C (3 days)	1.22 ± 0.21	75.4 ± 7.8	0.83 ± 0.08	0.09 ± 0.01
D (3 days)	1.72 ± 0.42	60.0 ± 3.4	0.85 ± 0.15	0.08 ± 0.02
C (17 days)	1.22 ± 0.05	69.4 ± 3.9	0.75 ± 0.04	0.07 ± 0.02
D (17 days)	2.27 ± 0.13*	48.4 ± 2.4	0.53 ± 0.03**	0.07 ± 0.01
C (30 days)	1.23 ± 0.07	72.2 ± 4.0	0.81 ± 0.06	0.08 ± 0.02
D (30 days)	5.10 ± 0.13***	22.4 ± 1.0**	0.45 ± 0.01**	0.08 ± 0.01
C (72 h)	1.13 ± 0.16	69.8 ± 2.67	0.77 ± 0.11	0.10 ± 0.02
S (72 h)	1.67 ± 0.20	38.4 ± 3.00**	0.37 ± 0.05*	0.07 ± 0.01*

C = Control; D = Diabetic; S = Starved
All values are expressed per mg protein and means ± SEM for 5 rats
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Table 4. Changes in activities of the enzyme in diabetic and starved rats liver

	Pyruvate kinase	Phosphofructo kinase	Aldolase	Glyxalase I
Control	0.35 ± 0.03	0.007 ± 0.001	0.02 ± 0.005	0.48 ± 0.06
Diabetic				
3 days	0.30 ± 0.01	0.006 ± 0.002	0.02 ± 0.005	0.48 ± 0.09
17 days	0.07 ± 0.01**	0.010 ± 0.002	0.03 ± 0.008	0.38 ± 0.10
30 days	0.13 ± 0.05*	0.008 ± 0.002	0.03 ± 0.004	0.49 ± 0.05
Starved				
72 h	0.20 ± 0.14*	0.011 ± 0.001	0.03 ± 0.002	0.45 ± 0.01

All values are presented as μmol/min per mg protein and means ± SEM for 5 rats
* $P < 0.05$; ** $P < 0.001$

Table 5. Urinary excretion of D- and L-lactate in diabetic, starved, and normal rats

	D-Lactate (nmol/ml)	L-Lactate (μmol/ml)	L-Lactate / D-Lactate
Diabetic	30 ± 8.0*	2.4 ± 0.8	80
Starved	120 ± 25.3**	37.2 ± 4.5**	310
Control	10.5 ± 5.7	4.8 ± 1.3	457

Values are means ± SEM for 5 rats
* $P < 0.01$; ** $P < 0.001$

Discussion

As far as we know, this paper is the first to report in detail D-lactate and MG contents in blood plasma and tissues of diabetic and starved animals. Some papers about L-lactate and pyruvate contents in such animals have been published

[2, 7, 9]. Although these papers provide some evidence, in biochemical texts it is not mentioned whether L-lactate and pyruvate levels become elevated in blood plasma and tissues of diabetic and starved animals. This may be due to contradictory results, which arise from inaccurate assay methods. From the present results, it can be said that the L-lactate level in blood plasma, liver, and skeletal muscle of starved rats fell significantly and a marked reduction of pyruvate was observed in them. Similarly, in diabetic rats, pyruvate level decreased in plasma, liver, and muscle to a great extent.

It is thought that the marked fall in pyruvate in liver of diabetic and starved rats may be first of all attributed to gluconeogenesis, i.e., pyruvate must be used as substrate for pyruvate carboxylase in these conditions. Secondly, the reduction in pyruvate concentration is probably due to the drastic decrease in hepatic pyruvate kinase activity after starvation for 72 h and after the 17th and 30th days of induced diabetes (Table 4). The authors had thought until this work that plasma levels of pyruvate and L-lactate in diabetic or starved state were always higher than in normal animals, because these metabolites were transported to the liver to reform glucose (Cori cycle).

Increased concentrations of glycerol in the liver during diabetes and starvation are widely acknowledged to be due to enhanced lipolysis (Table 2).

The reason why Pi level in liver and plasma elevated markedly in diabetic and starving rats cannot be clearly given here. However, it can be accounted for by the lowering of the rates of glycolysis and the pentose phosphate shunt, in which the formation of intermediates requires a large amount of phosphate and by an increase in the MG bypass activity, which will be discussed below. Wieland's group reported the level of Pi occurring during incubation both in the intact hepatocytes and the surrounding medium. In the absence of glucagon a considerable amount of Pi was released into the incubation medium. However, glucagon diminished the release of Pi. The group discussed the role of Pi in isolated hepatocytes, especially in mitochondria [21]. It cannot be explained now anyway whether the fact that Pi levels in plasma and liver elevated markedly is a result of carbohydrate metabolism or hormonal regulation.

The last problem is the formation of D-lactate or, rather, the physiological meaning of the MG bypass. As shown in Fig. 1, 2 mol of ATP was obtained by the conversion of glycerlaldehyde-3-phosphate to L-lactate, whereas the route from triose phosphate to D-lactate via MG (i.e., the MG bypass) did not yield any energy equivalent, but rather 1 mol of Pi. The MG bypass is energetically less advantageous than the EM pathway. To learn why triose phosphates flow into the MG bypass, we measured the enzyme activities of phosphofructokinase, pyruvate kinase, and glyoxalase I in livers of diabetic and starved rats, and concluded that glycolysis from glucose to pyruvate is regulated by three allosteric enzymes and another regulatory enzyme, glucokinase (Table 4). In this Table, aldolase is also listed, because MG is formed from triose phosphates [18]. As shown in Table 4, no marked differences between diabetic or starving and normal animals were found except in pyruvate kinase. Gunn and Taylor reported glucokinase and pyruvate kinase activities in liver decreased markedly in starving and diabetic animals, whereas the activity of phospofructokinase alters little in these animals [8]. According to Greenbaum, only the levels of fructose-1,6-bisphosphate increased and other glycolytic metabolites decreased in liver of diabetic, starving animals [7]. For these reasons, it is thought that triose phosphates derived from fructose-1,6-bisphosphate flow favorably into the MG bypass.

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