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CONSTITUTIVE HEPATIC GLUCOKINASE ACTIVITY IN *db/db* AND *ob/ob* MICE

TERENCE T. YEN and NANCY B. STAMM

Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, IN 46285 (U.S.A.)

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Summary

The specific activity of hepatic glucokinase (ATP: D-glucose 6-phosphotransferase, EC 2.7.1.2) in *db/db* mice and *ob/ob* mice was higher than in normal mice. All enzymes had a similar K_m and, thus, the difference in activity was not due to differences in the affinity of enzyme molecules to substrates. Mixing liver extracts with high or low enzyme activities yielded additive results, as expected, which ruled out the involvement of an inhibitor or activator of the enzyme. Fasting normal mice of either strain for three days decreased glucokinase activity. However, fasting *db/db* or *ob/ob* mice for as long as 10 days had no effect on enzyme activity, indicating that glucokinase in *db/db* or *ob/ob* mice was out of regulation or constitutive. The constant, abnormally high glucokinase activity may be a contributing factor to the obesity of *ob/ob* or *db/db* mice. These mice provide a model system to study the regulation of this rate-limiting enzyme of glucose metabolism.

Introduction

Glucokinase (ATP : D-glucose 6-phosphotransferase, EC 2.7.1.2), an enzyme that phosphorylates glucose in the liver, is characterized by a high K_m for glucose [1,2] and is affected by various hormonal and nutritional states. The activity of glucokinase is low in fasted animals [3—11] and alloxan-diabetic animals [3—7,9,10]; enzyme activity can be restored by refeeding starved animals [7,8,10,11] or by administering insulin to alloxan-diabetic animals [5,7,9,10]. Both glucose and insulin are essential for the restoration of enzyme activity [7,11,12] or the development of glucokinase activity in neonatal rats [10]. The reappearance of glucokinase upon refeeding or insulin administration involves the induction of de novo protein synthesis [5,7,11].

Glucokinase activity is elevated in genetically obese mice (*ob/ob* mice [13, 14] or *db/db* mice [15,16]). The reason for this heightened activity is not clear. In the present experiments we have further studied this enzyme in *ob/ob* and *db/db* mice; these studies included the determination of the kinetic properties of the enzyme, the mixing of enzymes with high or low activities, and the effect of fasting.

Materials and Methods

Mice. Male C57BL/KsJ-normal and *db/db* mice and C57BL/6J-normal and *ob/ob* mice were supplied by the Jackson Laboratory, Bar Harbor, Maine. They were housed in transparent plastic cages and fed water and Purina Rodent Laboratory Chow 5001 ad libitum except in the fasting experiments where only water was available. The ambient temperature of the animal room was maintained at about 25°C. The lights in the room were on from 6 a.m. to 6 p.m.

Preparation of liver extracts. Mice were killed by decapitation. About 1 g liver was homogenized in 3 ml of a cold solution containing 150 mM KCl/5 mM disodium EDTA/5 mM $MgCl_2$/9.3 mM mercaptoethanol, pH 7.0. The volume of the homogenate was made up to 10% (w/v) with the same solution, and the homogenate was centrifuged at 1500 × *g* for 10 min in a Sorvall RC-2B centrifuge. The supernatant was then centrifuged at 100000 × *g* for 45 min in a Spinco ultracentrifuge. The supernatant obtained from the second centrifugation was used in the glucokinase assay.

Glucokinase assay. The glucokinase reaction product, glucose 6-phosphate, was oxidized by excess glucose-6-phosphate dehydrogenase and NADP and the formation of NADPH was monitored optically [8]. The assay mixture contained in a total volume of 2 ml: 20 mM histidine-HCl/20 mM Tris/2 mM disodium EDTA/12 mM $MgCl_2$/12 mM ATP/25 mM NADP/0.2 units of yeast glucose-6-phosphate dehydrogenase (Sigma)/50 mM glucose/0.05 ml of the liver extract, unless otherwise specified. The pH of the mixture of histidine-HCl, Tris and EDTA was adjusted to 8.0 with NaOH so that the final pH of the assay mixture measured about pH 7.3. The temperature of the reaction was maintained at 37°C. The absorbance of the reaction mixture was monitored for 7 min at 340 nm with a Beckman Model 25 spectrophotometer. The difference in absorbance between the end of the 7th min and the beginning of the 4th min was used to calculate the amount of NADPH formed, using $6.22 \cdot 10^3$ as the molar extinction coefficient. The activity of glucokinase is expressed as nmol NADPH formed/min per mg protein or μmol NADPH formed/min per g liver, except in the mixing experiments where activities are expressed as nmol NADPH formed/min per ml reaction mixture. The amount of NADPH formed due to the presence of endogenous 6-phosphogluconate dehydrogenase, in the extract, was tested experimentally by substituting 6-phosphogluconate for glucose as substrate in a concentration that assumed that half of the NADPH generated in the overall reaction was from this enzyme. Under such conditions, no more than 15% and 22% of NADPH in the reaction of lean and mutant mouse extract, respectively, was produced by the endogenous 6-phosphogluconate dehydrogenase.

The activity of hexokinase accounted for less than 3% of the activity of glucokinase in extracts from normal or mutant mice and was thus considered negligible.

The optimum concentration for ATP was 12 mM. Higher concentrations of ATP were inhibitory to the reaction.

K_m values were calculated according to the method of Wilkinson [17].

Other assays. Protein was determined on the supernatants by the method of Lowry et al. [18] using Sigma Protein Standard Solution (Sigma No. 540-10) as the standard.

Glucose concentration of the whole blood was measured by Glucostat (Worthington) using dextrose (Mallinckrodt) as the standard.

Plasma insulin was measured according to the method of Makulu et al. [19] using porcine insulin as the standard. The sensitivity of the assay was 0.5—10 μunits. Between 10 to 25 μl plasma were assayed.

Hepatic glycogen was assayed as follows: 50—100 mg liver were digested in 30% KOH, in a boiling water bath for 30 min. After the addition of 10% $ZnSO_4$ and 95% ethanol, a precipitate formed which was collected by centrifugation. This precipitate was hydrolyzed in 1 N HCl for 2 h in a boiling water bath. The pH of the hydrolysate was then neutralized with NaOH. The amount of glucose in the hydrolysate was determined by Glucostat. Rabbit liver glycogen (Sigma, type III) was used as the standard.

Results

The *db/db* mice were hyperglycemic and hyperinsulinemic at all three ages, but their concentrations of hepatic glycogen were normal (Table I).

The specific activity of glucokinase, expressed as activity per mg protein or per unit weight of liver, was higher in the livers of *db/db* mice than in normal mice at all three ages (Table I). The higher specific activity based upon protein was not due to a difference in the protein content of the liver. The older *db/db* mice, 19—25 weeks of age, had 10% less protein/g liver, but that difference could not account for the doubling of specific activity.

Similar to *db/db* mice, *ob/ob* mice were also hyperinsulinemic, had normal hepatic glycogen content and higher specific activities of glucokinase than C57BL/6J-normal mice (Table I). However, *ob/ob* mice were normoglycemic.

C57BL/6J-normal mice had higher specific activity of glucokinase than C57BL/KsJ-normal mice (Table I). Coleman [20] concluded that the variation of glucokinase activity among strains of mice is controlled by a single gene.

For each substrate, glucose or ATP, the K_m of glucokinase was found to be similar between the mutant mouse and its corresponding normal mouse (Table II) suggesting that higher glucokinase activity is not due to differences in the affinity of enzyme molecules to substrates.

Extracts of *db/db* and normal mice and extracts of *ob/ob* and normal mice were mixed in different proportions (Table III) in order to determine if the difference in enzyme activities might be due to the presence of an activator or inhibitor. The activities of the mixed extracts were additive, which ruled out the involvement of an activator or inhibitor.

When normal mice were fasted for 16 h, glucokinase activity did not change,

TABLE I

BODY AND LIVER WEIGHT, BLOOD GLUCOSE AND PLASMA INSULIN CONCENTRATION, HEPATIC PROTEIN AND GLYCOGEN CONTENT AND HEPATIC GLUCOKINASE ACTIVITY OF C57BL/KsJ-NORMAL AND DIABETIC (*db/db*) MICE AND C57BL/6J-NORMAL AND OBESE (*ob/ob*) MICE

All data are expressed as mean ± S.E. (*N*).

Genotype	Body wt. (g)	Liver wt. (g)	mg protein **/g liver	Glucokinase		Blood glucose mg/dl	mg glycogen/g	Plasma insulin (μunits/ml)
				nmol/min per mg protein	μmol/min per g liver			
Age: 4.5—6 weeks								
+/+ (12)	17.1 ± 0.3	1.01 ± 0.03	74 ± 7	53 ± 3	3.87 ± 0.23	129 ± 4	42.4 ± 3.4	<20
db/db (12)	26.6 ± 1.8	1.81 ± 0.15	73 ± 4	107 ± 8	7.49 ± 0.30	199 ± 27	43.6 ± 3.3	1125 ± 194
p *	<0.0005	<0.0005	n.s.	<0.0005	<0.0005	<0.01	n.s.	
Age: 14—15 weeks								
+/+ (8)	23.8 ± 0.6	1.11 ± 0.07	80 ± 6	39 ± 3	3.04 ± 0.21	115 ± 6	37.7 ± 5.7	<20
db/db (8)	57.4 ± 1.1	3.13 ± 0.23	69 ± 5	84 ± 5	5.63 ± 0.10	392 ± 14	40.8 ± 2.2	811 ± 276
p	<0.0005	<0.0005	n.s.	<0.0005	<0.0005	<0.0005	n.s.	
Age: 17—28 weeks								
+/+ (4)	26.5 ± 0.5	1.30 ± 0.01	91 ± 2	38 ± 3	3.41 ± 0.21	138 ± 10	43.8 ± 6.8	<20
db/db (19)	59.6 ± 1.5	3.15 ± 0.14	82 ± 3	78 ± 5	6.21 ± 0.31	412 ± 18	39.0 ± 5.7	292 ± 98
p	<0.0005	<0.0005	<0.025	<0.0005	<0.0005	<0.0005	n.s.	
+/+ (10)	31.4 ± 0.7	1.47 ± 0.04	71 ± 3	73 ± 2	5.18 ± 0.19	157 ± 4	26.9 ± 5.2	<20
ob/ob (6)	59.1 ± 0.8	3.39 ± 0.22	76 ± 2	126 ± 9	9.59 ± 0.79	148 ± 19	16.2 ± 3.5	1036 ± 236
p	<0.0005	<0.0005	n.s.	<0.0005	<0.0005	n.s.	n.s.	

* According to Student's *t*-test.

** 100 000 × *g* supernatant protein.

n.s., not significantly different at $P = 0.05$.

TABLE II

K_m VALUES FOR GLUCOSE AND ATP OF GLUCOKINASE FROM C57BL/KsJ-*db/db*, C57BL/6J-*ob/ob* AND CORRESPONDING NORMAL MICE

K_m values, calculated according to the Wilkinson method [17], are expressed as mean ± S.E. in nM. The number in parenthesis indicates the number of determinations made to obtain that mean. Each determination used a different extract, and each extract was prepared from the liver of a different mouse. Thus, for each K_m value, at least three determinations were made on three extracts prepared from three different mice of the same genotype. The ages of mice varied but no difference of K_m value was observed due to age. The concentrations used to determine the K_m value for glucose varied from 5 to 100 mM and those used to determine the K_m value for ATP varied from 0.6 to 6.0 mM. n.s. indicates no significant difference at $P = 0.05$ according to Student's *t*-test

	Glucose	ATP
C57BL/KsJ +/+	12.3 ± 0.4 (3)	0.92 ± 0.11 (4)
db/db	11.4 ± 1.4 (3)	0.85 ± 0.07 (3)
P	n.s.	n.s.
C57BL/6J +/+	15.0 ± 2.1 (3)	1.05 ± 0.01 (3)
ob/ob	12.7 ± 0.2 (3)	1.25 ± 0.13 (4)
P	n.s.	n.s.

however, fasting for 3 days produced a 53% decrease in the enzyme activity in the C57BL/KsJ mice and a 22% reduction in the C57BL/6J mice. In contrast, fasting *db/db* or *ob/ob* mice for as long as 10 days had no effect on glucokinase activity in spite of a normalization of blood glucose concentrations and a reduction in hepatic protein and glycogen content (Table IV). The majority of the fasted *db/db* and *ob/ob* mice had plasma insulin levels less than 20 μU/ml which was below the sensitivity of our insulin assay.

TABLE III

MIXING OF LIVER SUPERNATANTS FROM NORMAL AND *db/db* MICE AND NORMAL AND *ob/ob* MICE : GLUCOKINASE ACTIVITY

0.05 ml of each 0.2 ml mixture was assayed.

ml of Extracts (+/+)		Glucokinase activity (nmol/min per ml reaction mixture)	
		Observed	Expected
db/db			
0.20 (100%)	0	20.6	—
0.18 (90%)	0.02 (10%)	18.3	19.3
0.14 (70%)	0.06 (30%)	17.0	16.8
0.10 (50%)	0.10 (50%)	14.5	14.2
0.06 (30%)	0.14 (70%)	10.3	11.7
0.02 (10%)	0.18 (90%)	9.8	9.1
0	0.20 (100%)	7.8	—
ob/ob			
0.20 (100%)	0	14.9	—
0.18 (90%)	0.02 (10%)	14.1	14.3
0.14 (70%)	0.06 (30%)	14.2	12.7
0.10 (50%)	0.10 (50%)	11.6	11.3
0.06 (30%)	0.14 (70%)	8.7	9.8
0.02 (10%)	0.18 (90%)	8.8	9.2
0	0.20 (100%)	7.6	—

TABLE IV

EFFECT OF FASTING ON BODY AND LIVER WEIGHT, BLOOD GLUCOSE CONCENTRATION AND HEPATIC PROTEIN AND GLYCOGEN CONTENT

All data are expressed as mean ± S.E. (*N*). mg protein/g liver, 100 000 × *g* supernatant protein.

	Mean weight loss (g)	Liver weight (g)	mg protein/g liver	mg glucose/dl blood	mg glycogen/g liver	Glucokinase (nmol/min per mg protein)
C57BL/KsJ mice						
db/db: fed (19)	0	3.15 ± 0.14	82 ± 3	412 ± 18	39.0 ± 5.7	78 ± 5
3 day fasted (8)	5.5	2.10 ± 0.11 [e]	100 ± 5 [d]	152 ± 29 [e]	15.1 ± 0.2 [e]	71 ± 7
5 day fasted (4)	8.3	1.84 ± 0.10 [e]	90 ± 2 [b]	128 ± 17 [e]	28.4 ± 6.8	86 ± 3
7 day fasted (4)	7.2	1.66 ± 0.12 [e]	65 ± 6 [c]	200 ± 27 [e]	16.7 ± 3.9 [d]	73 ± 2
10 day fasted (4)	11.7	1.64 ± 0.08 [e]	81 ± 5	170 ± 17 [e]	14.0 ± 3.1 [e]	96 ± 11
+/+: fed (4)	0	1.30 ± 0.01	91 ± 2	138 ± 10	43.8 ± 6.8	38 ± 3
16 h fasted (4)	1.5	0.97 ± 0.03 [e]	88 ± 4	108 ± 8 [a]	20.3 ± 1.6 [c]	42 ± 1
3 day fasted (4)	4.5	1.02 ± 0.03 [e]	97 ± 2 [a]	55 ± 2 [e]	2.0 ± 0.8 [e]	18 ± 1 [e]
C57BL/6J mice						
ob/ob: fed (6)	0	3.39 ± 0.22	76 ± 2	148 ± 19	16.2 ± 3.5	126 ± 9
3 day fasted (4)	9.8	3.29 ± 0.06	58 ± 2 [e]	118 ± 11	4.6 ± 0.7 [c]	152 ± 6 [b]
7 day fasted (4)	14.3	2.26 ± 0.14 [d]	66 ± 5	113 ± 9	4.1 ± 1.8 [c]	111 ± 10
10 day fasted (4)	21.2	2.33 ± 0.22 [c]	59 ± 5 [c]	60 ± 8 [d]	10.4 ± 7.3	109 ± 14
+/+: fed (10)	0	1.47 ± 0.04	71 ± 3	157 ± 4	26.9 ± 5.2	69 ± 2
16 h fasted (4)	3.1	1.12 ± 0.07 [e]	74 ± 1	94 ± 7 [e]	0.2 ± 0.2 [e]	72 ± 5
3 day fasted (4)	7.6	1.17 ± 0.07 [d]	72 ± 5	94 ± 8 [e]	0.4 ± 0.3 [e]	53 ± 7 [a]

Significantly different from corresponding fed mice: [a] $P < 0.05$; [b] $P < 0.025$; [c] $P < 0.01$; [d] $P < 0.005$; [e] $P < 0.0005$.

Discussion

In contrast to alloxan-diabetic animals which had very little glucokinase activity [3—7,9,10], the genetically obese and diabetic *db/db* and *ob/ob* mice had higher glucokinase activity than corresponding normal mice. At the ages studied, both *db/db* mice and *ob/ob* mice were hyperinsulinemic. They were fasted for as long as 10 days so that the influence of hyperinsulinemia could be determined. This period of fasting normalized their plasma insulin and glucose levels, but hepatic glucokinase activity remained high.

The additive results obtained by mixing extracts with high or low glucokinase activities ruled out the involvement of an activator or inhibitor of the enzyme. Thus, the differences in the activity must reside in the enzyme itself; either the enzyme molecules differed in efficiency or the extract with higher activity had more enzyme molecules present. The fact that glucokinase from all samples had similar K_m values suggests that the enzymes were not qualitatively different, but rather differed in quantity. Coleman [20] concluded, after measuring the glucokinase activity in seven strains of mice and conducting a genetic study between one strain of high activity and one strain of low activity, that the variations in glucokinase were attributed to a single gene. The rates of heat denaturation of the enzyme from high activity and low activity strains were not different, a fact which also indicates a quantitative but not a qualitative difference. Thus, the 'glucokinase' gene probably is a regulatory rather than a structural gene.

Nutritional and hormonal changes affect the activity of glucokinase by altering the synthesis of enzyme protein [21], but the specific mediator and mechanism have not been identified. Insulin and glucose act as inducers and glucocorticoids were reported to have a permissive effect [9,11,22—26]. On the other hand, epinephrine, norepinephrine, isoproterenol, glucagon, cyclic AMP and dibutyryl cyclic AMP inhibited the restoration of glucokinase activity by glucose refeeding [11,27]. By placing intact or adrenalectomized rats on various diets, Seitz et al. [26] demonstrated that there was an inverse relationship between glucokinase activity and hepatic cyclic AMP concentration. The constitutive nature of glucokinase in *ob/ob* and *db/db* mice makes these mice an ideal system in which to study the regulation of this enzyme and to identify the mediator that governs the synthesis of enzyme protein. We attempted to normalize the glucokinase activity in *ob/ob* mice by increasing the level of hepatic cyclic AMP with glucagon administration, however, glucagon failed to raise hepatic cyclic AMP concentration in these mice (data reported elsewhere).

The uptake and phosphorylation of glucose in liver are concentration-dependent and controlled by glucokinase [21]. The constantly high glucokinase activity in *db/db* and *ob/ob* mice coupled with a permanent hyperglycemia in *db/db* mice and a transient hyperglycemia during the growing phase in *ob/ob* mice, makes glucose available at all times as a substrate for lipogenesis. The contribution of high glucokinase activity to enhanced hepatic lipogenesis in these mice [28] is under further analysis.

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