Vol. 106, No. 2, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1982 Pages 379-384

EFFECT OF STREPTOZOTOCIN-INDUCED DIABETES ON THE TURNOVER OF HEXOKINASE II IN THE RAT*

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Received March 31, 1982

SUMMARY. Skeletal muscle hexokinase II activity and turnover rates were measured in the normal and streptozotocin-induced diabetic rat. Enzyme activity decreases in the diabetic animal relative to the normal rat; however, the specific activity of hexokinase II is essentially the same for the two conditions. No alteration is observed in the relative rate of hexokinase II synthesis in the normal or diabetic rats, but there is a 3-fold increase in the rate of hexokinase II degradation in the latter group of animals. These results suggest that the primary cause of the well-established decrease in hexokinase II activity in skeletal muscle of the diabetic is an increase in the rate of enzyme degradation.

INTRODUCTION. The skeletal muscle of the rat (1,2) and of other species (3) contains three molecular forms of hexokinase, designated I, II, and III (2). Several investigators have shown that the hexokinase II activity in skeletal muscle decreases in the diabetic state (2,4). The cause of this decrease has not been determined.

Katzen et al. (5) studied the association of hexokinase II with soluble and particulate fractions of skeletal muscle from normal and streptozotocin-induced diabetic rats. Using starch-gel electrophoresis and DEAE-cellulose chromatography, Katzen and his colleagues found that the amount of hexokinase II associated with all fractions of the gastocenemius muscle was much less than normal in the diabetic rat. They also showed that the loss of hexokinase II activity was not due to inhibition by glucose 6-phosphate or other metabolites. They suggested that the loss of activity was due to a decrease in the amount of enzyme.

*This research was supported in part by Research Grant 10645 from the National Institutes of Health and by Grant PCM-8101999 from the National Science Foundation. Journal Paper No. J-10607 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Ia. 50011, Project 2244.

0006-291X/82/100379-06$01.00/0

The loss of hexokinase II activity in the diabetic state could be due to the loss of stabilization factors, as suggested by Rose and Warms (6), who recently investigated the stability of hexokinase II in Ehrilich-Lettre hyperdiploid ascities cells. They found that the temperature-sensitive hexokinase II could be stabilized by K^+ with no effect on the kinetic properties of hexokinase II and that the thermal stability of the enzyme was further increased by the addition of both K^+ and glucose. Based on these findings, they suggest that the changes in the K^+ and glucose levels that occur in the diabetic state could contribute to the decrease in hexokinase II in insulin-sensitive tissues.

Several investigators have shown that overall protein synthesis is decreased in skeletal muscle of the diabetic rat (7,8). This suggests the possibility that the decrease in hexokinase II activity could be due to a decrease in protein synthesis.

Studies on the rate of degradation of hexokinase II in normal and streptozotocin-induced diabetic rats were undertaken to gain insight into the cause(s) for the decrease of hexokinase II activity in the diabetic state.

MATERIALS

Male Wistar rats were obtained from Hill Top. L-[U-^{14}C]leucine (300Ci/mol) and L-[4,5-^{3}H]leucine (61Ci/mmol) were purchased from Amersham. Protosol was purchased from New England Nuclear.

Staphyloccus aureus and all materials from ELISA were purchased from Sigma. Distilled,deionized water was used in the preparation of all reagents. All other chemicals were of the highest purity available commercially.

METHODS

Hexokinase II was purified from rat skeletal muscle by the procedure of Qadri and Easterby (9) to a specific activity of 176 units/mg. The enzyme was judged pure by gel electrophoresis, and the isozymic identity confirmed by kinetic analysis.

Antibody Production- Antibodies to hexokinase II were raised in a New Zealand white rabbit by subcutaneous injection of 200µg hexokinase II in Freunds' incomplete adjuvant. Additional injections were made 14, 28, and 60 days later. Antisera was obtained 7 days after the final booster. Thin-layer Ouchterlony double-diffusion analysis was carried out on the antisera on 1% agarose plates(10). The antisera gave a single precipitin line against pure hexokinase II and against crude extract. No precipitin line against pure hexokinase I was observed.

Immunoprecipitation Reaction-Skeletal muscle dissected from the hind legs of male Wistar rats was extracted by homogenization for 2 min. in extraction buffer (0.05M Tris-Cl, 0.15M NaCl, 0.005 M EDTA, 0.1 M glucose, 0.05% Triton X-100, pH 7.4). Fifty µl sera from an untreated rabbit and

100 µl of a Staphyloccus aureus suspension (0.05g S. aureus in extraction buffer without glucose) were added to a 300 µl aliquot of the enzyme containing homogenate (11). The samples were incubated for 30 min. at 0°C and then centrifuged 5 min. (3000 x g). Twenty-five µl anti-rat hexokinase II were added to the supernatant fluid, and the samples were incubated for 8 hours at 0°C. S. aureus suspension (200 µl) was added to the samples which were then incubated for 2 hours at 0°C. Samples were centrifuged 5 min. (3000 x g), and the pellet washed 3 times with cold 0.15 M NaCl and 0.05% Triton X-100. The immunoprecipitate was incubated in 400 µl protosol for 24 hours at 37°C, and the solution transferred to a scintillation vial. After adding 15 ml of a toluene scintillation mixture (PPO, bismethyl POPOP), radioactivity was counted on a Packard liquid scintillation counter. Channels were chosen so that no ^{3}H radioactivity was in the ^{14}C channel and about 18% of the ^{14}C radioactivity was in the ^{3}H channel. Sufficient radioactivity was counted to give a counting error of 4% or less. All measurements were done in triplicate.

Protein in a 300 µl aliquot was precipitated with 12% trichloroacetate and washed according to the procedure of Siekevitz(12). The precipitate was digested in 400 µl Protosol for 24 hours at 37°C, and the sample treated as indicated above.

Enzyme Activity-Enzyme activity in the extracts was determined at pH 8 with 20 mM HEPES buffer, 3mM glucose, 10 mM ATP, and 10 mM $MgCl_2$ in a glucose-6-P dehydrogenase coupled assay(13). Amount of hexokinase II was determined using ELISA(14). A standard curve was included on each plate, and each sample was analyzed in triplicate.

Animals- Male Wistar rats weighing 180-200g were used in the study. The diabetic state was induced by injection of 75 mg streptozotocin/100g of body weight after 24 hours of fasting. Rats were used as diabetic if the blood glucose levels were above 400mg/100 ml.

Measurement of Enzyme Degradation- The fractional turnover rate of hexokinase II in normal and in diabetic rat skeletal muscle was estimated using a double-isotope method as described by Arias et al. (15) and Glass and Doyle(16). At zero time ($t \approx 0$ hour) [^{14}C]leucine (13µCi/100 g of body weight) was injected. Additional injections of [^{3}H]leucine (26µCi/100 g of body weight) were given 12, 24, and 36 hours later. After one hour of exposure to the [^{3}H]-label, the rats were sacrificed, and procedures followed as described above.

From the estimated $^{3}H/^{14}C$ ratios measured in the hexokinase II immunoprecipitate, the rate constant of degradation was calculated according to the following equation(17):

$$K_d\ (hr^{-1}) = \frac{2.303}{t-t_o} \times \log \left[{}^{3}H/{}^{14}C\right]_{t_o} \Big/ \left[{}^{3}H/{}^{14}C\right]_{t} \qquad (1)$$

The apparent half-life of the hexokinase II was calculated from the following relationship:

$$t_{1/2} = \ln 2/K_d \qquad (2)$$

RESULTS AND DISCUSSION

Figure 1 illustrates the rates of degradation of hexokinase II in the untreated and streptozotocin-treated diabetic rat. It can be seen from the graphs that the degradative rate of hexokinase II is increased in the diabetic animal. The relative rate of synthesis of hexokinase II was determined at one hour after injection of [^{14}C]leucine and found to be the same for the diabetic and normal rats.

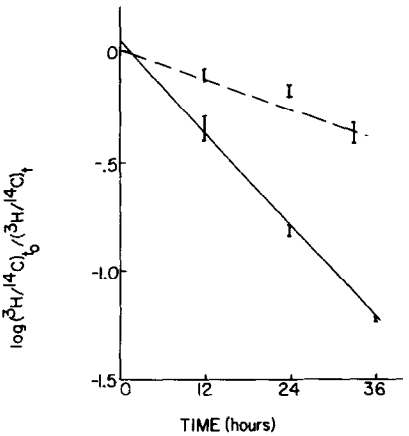

Figure 1. Plot of $Log(^3H/^{14}C)_{t_o}$ $/(^3H/^{14}C)_t$ versus time for skeletal muscle hexokinase II in normal(– –), and diabetic(——) rats. Lines are least-squares computer fits to the data.

The estimated rate constant of degradation, K_d, for hexokinase II is greater in the diabetic state, by a factor of 3, than in the normal state (K_d= 0.069 hr $^{-1}$ and K_d= 0.023 hr $^{-1}$,respectively) as shown in Table I. The incorporation of [^{14}C]leucine and [^{3}H]leucine into the cytosolic proteins was measured for these same samples, and the results can be found in Table I.

Table I

Protein Degradation in Diabetic and Normal Rats*

Rats	Time (Hr)	No. of Rats	Hexokinase II ^3H dpm / mg muscle	Hexokinase II ^{14}C dpm / mg muscle	Hexokinase II $^3H/^{14}C$	Hexokinase II K_d (Hr^{-1})	Cytosolic Protein $^3H/^{14}C$
Normal							
	0	4	3.91±0.36	4.93±1.11	0.89±0.11		3.28±0.54
	12	4	3.40±1.20	2.85±0.33	1.19±0.03	0.024	3.39±1.30
	24	4	1.77±0.48	0.75±0.20	1.40±0.01	0.019	5.05±0.42
	36	4	2.83±0.73	1.06±0.44	2.25±0.51	0.026	4.54±1.50
Diabetic							
	0	4	3.30±1.10	2.84±0.86	1.12±0.18		2.73±0.56
	12	4	2.66±0.28	1.24±0.70	2.14±0.62	0.054	3.57±0.87
	24	3	2.66±0.42	0.41±0.05	6.76±0.91	0.075	4.22±0.35
	36	3	3.51±1.10	0.16±0.06	18.57±0.10	0.078	2.14±0.25

* $^3H/^{14}C$ ratios in immunoprecipitable hexokinase II and cytosolic protein and the rate constant of degradation, K_d, in skeletal muscle of diabetic and normal rats. See "Materials and Methods" for details.

Table II

Specific Activity of Hexokinase II in Diabetic and Normal Rats*

Rat Time (Hr)	mUnits enzyme activity / mg muscle	mg hexokinase II / mg muscle	Units enzyme activity / mg hexokinase II
Normal			
12	0.87±0.10	$2.76\times10^{-6}\pm0.64\times10^{-6}$	336±96
24	0.81±0.17	$1.08\times10^{-6}\pm0.25\times10^{-6}$	671±192
36	0.68±0.04	$1.59\times10^{-6}\pm0.31\times10^{-6}$	418±123
Diabetic			
12	0.69±0.05	$1.30\times10^{-6}\pm0.12\times10^{-6}$	531±79
24	0.47±0.07	$7.24\times10^{-7}\pm0.15\times10^{-7}$	674±192
36	0.41±0.09	$1.79\times10^{-6}\pm0.33\times10^{-6}$	265±81

* Units enzyme activity/mg hexokinase II in diabetic and normal rats. The average value for the normal rat was 475 and for the diabetic rat 490, showing no difference between the normal and diabetic rat. For details see "Materials and Methods".

The amount of enzyme (ng) was determined for each sample and is expressed in Table II as mg enzyme/mg muscle. The "specific activity" was determined by dividing the units of enzyme activity/mg muscle by the mg hexokinase II/mg muscle. Although this does not represent the true specific activity of the hexokinase II, it does represent the relative change of the hexokinase II activity/mg hexokinase II since the levels of the hexokinase I should remain constant (2,4). Although the level of hexokinase III is thought to increase in diabetic skeletal muscle, our assay precludes measuring hexokinase III activity(18). As shown in Table II, this ratio does not change from the normal to the diabetic rat. This points to the conclusion that the specific activity of the hexokinase II is not affected.

It has been established by Schimke et al. (19) that the "quantity of an enzyme in animal cells is a balance between the rate of synthesis and the rate of degradation of the enzyme." As shown in this report, the relative rate of synthesis in normal and diabetic rats is the same, while the rate of degradation is higher in the diabetic than in the normal rat. The "specific activity" of the hexokinase II did not change from the normal to the diabetic state. These results suggest that there is some mechanism by which hexokinase II is rendered less stable in the diabetic

state. Whether this is due to the loss of stabilization factors or to some signal for increased degradation of the hexokinase II, such as phosphorylation of the enzyme, or, as suggested by Rose and Warms(6), oxidation of hexokinase II,is not known at this time.

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