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Biochemical Pharmacology, Vol. 30, pp. 98–101.

0006-2952/81/010098-04$02.00/0

Liver fructose bisphosphatase concentration and activity in alloxan induced diabetes

(*Received* 24 *May* 1980; *accepted* 14 *August* 1980)

In a previous publication, we reported on the development and application of a radioimmunoassay for rabbit liver and kidney fructose bisphosphatase (EC 3.1.3.11) [1]. A 2- to 3-fold increase in concentration was observed following the onset of alloxan diabetes. A proportional increase in activity also occurred so that the specific activity remained unchanged in the untreated, acutely diabetic rabbit. In the present study, we elaborate on the influence on this enzyme of both untreated and insulin-treated diabetes. The application of a specific radioimmunoassay allowed us to measure the concentration of the enzyme independently of its activity.

Forty-five New Zealand rabbits weighing approximately 1200 g each and of both sexes were used in this study. All rabbits were maintained on a conventional, commercial rabbit chow diet until they were killed. Diabetes was induced by a single intravenous injection of alloxan (100 mg/kg) administered under ketamine hydrochloride and promazine anesthesia. Diabetes was established, usually at 72 hr, by the finding of a blood glucose value of approximately 500 mg/dl or of massive glucosuria. Such animals, if left untreated beyond 72 hr, died within the next few days. Lente insulin (U-40) was obtained from the Eli Lilly Co., Chicago, IL. Preparation and evaluation of goat gamma-globulin against rabbit liver frutose bisphosphatase and the maximum activity assay at 24° have been described [1]. Radioiodination of fructose bisphosphatase was accomplished using a modification of the method of Bolton and Hunter [2]. The ester was iodinated to a specific activity of about 5 mCi/μg. The enzyme was labeled to the extent of approximately 12 ^{125}I atoms/tetramer. Radioimmunoassay of fructose bisphosphatase was done as described [1] and utilized rabbit anti-goat IgG gamma-globulin as the second stage precipitation system. Animals were anesthetized and exsanguinated and the livers were removed and weighed. Homogenates (20%, w/v) were prepared and centrifuged at 115,000 *g* for 60 min. For activity measurements 10 μl of each supernatant fraction was assayed; the assay mixture contained an AMP-removal system [1]. It was established for even the most active extracts that 10 μl never exceeded the capacity of the assay to measure V_{max}. For enzyme concentration measurements, 1:10,000, 1:1,000 and 1:100 dilutions of the 115,000 *g* cytosol preparation were made with 1% egg albumin buffer. Twenty and 50 μl aliquots of each dilution were analyzed by radioimmunoassay.

Homogeneous enzyme for iodination and for use as a standard in the radioimmunoassay procedure was prepared from frozen livers of young animals, as described by Ulm *et al.* [3]. The enzyme had a specific activity of 17 units/mg at 24° (2.5 units/nmole). When the purified enzyme was maintained at 100° in 8 M urea and 1% dodecylsulfate for 5 min and submitted to SDS gel electrophoresis, even 48 μg gave only one visibly stained band. Standard enzyme was assayed for concentration by a fluorescamine assay [4, 5]. Enzyme activity was localized in 3.65% polyacrylamide slab gels utilizing principles presented by Gabriel [6]. An overlay of 1% agar containing the fructose bisphosphatase assay mixture [2], meldola blue dye (2.5 mg/100 ml), and nitrotetrazolium salts (25 mg/100 ml) was prepared and allowed to develop in the dark. Control gels did not contain fructose bisphosphatase. Data are presented as the mean ± one standard deviation.

The specific activity of the purified enzyme (2.5 units/nmole) measured by the Lowry method for protein was almost the same as that of liver extracts from normally fed rabbits (2.6; see Table 1) measured by our radioimmunoassay. The excellent agreement argues against potential problems with the radioimmunoassay, such as interference by non-specific proteins.

The concentration of fructose bisphosphatase and the units of activity per gram of liver were above normal in the diabetic animals (Table 1, No. 2), which caused a net increase of 144 nmoles of enzyme (157 to 301) per liver and of 351 units of activity per liver (413 to 764). No change in specific activity occurred, because mass and activity both changed 2-fold. Neither variable returned to normal after treatment with insulin. Even after 10 days of daily insulin injection (Table 1, No. 6), enzyme content and activity continued to increase. Enzyme content was 9-fold greater than control values, but activity was only 4-fold greater. This resulted in a decrease in specific activity to 1.3 units/nmole. The situation was essentially the same after 15 days of insulin replacement therapy (Table 1, No. 7). During the time between alloxan administration and sacrifice, animals of groups 5, 6 and 7 had total body weight

gains of 15, 10 and 32 per cent respectively. Because insulin did not reverse the abnormal intracellular enzyme situation, four normal rabbits were injected with 2 units of the hormone daily for 5 days. One hour after the last injection the animals were killed and their livers were analyzed. The results were indistinguishable from those of group 1 (controls) (Table 1). Thus, insulin was not responsible for the increase in enzyme mass and the decrease in specific activity.

Good control of diabetes, as reflected in normal plasma glucose concentration, was difficult to achieve in rabbits given unrestricted access to food. For example, at sacrifice mean postprandial plasma glucose for group 6 animals was 409 mg/dl ± 91. However, it was only 156 mg/dl ± 57 for group 5 animals and 204 ± 60 for group 7. It was 186 ± 27 for control rabbits and 559 ± 79 for the untreated diabetics.

Analysis of the non-denaturing polyacrylamide slab gels, in which fructose bisphosphatase had been visualized specifically through activity staining, revealed no difference in the size of the fructose bisphosphatase from the liver extract of normal animals compared to that from diabetic or treated-diabetic animals.

Taunton *et al.* [7] showed that fructose bisphosphatase activity in rats is subject to rapid changes after hormone administration. The enzyme is known also to be inhibited by substrate [8] and by reaction with pyridoxal phosphate [9], ATP and ADP [10], and AMP. Inhibition by ATP or ADP may have been due to heavy metal ion contamination of those preparations [11]. Recently, evidence has been presented which indicates that there is an interaction between fructose bisphosphatase and fructose bisphosphate aldolase [12]. Such an interaction might be expected to influence reaction rates. The enzyme has been shown to be activated *in vitro* by long chain fatty acids [13]. Because rapid activation and inactivation phenomena may occur by covalent modification [14], it is uncertain that activity measurements directly reflect intracellular enzyme protein concentration in all cases or that direct proportionality exists between activity and concentration. We have addressed this issue by the application of a specific radioimmunoassay that measures the enzyme concentration directly and thereby provides an investigative dimension that was not available previously.

In the alloxan diabetic rabbit, the concentration of enzyme increased and continued to do so despite prolonged treatment with doses of insulin (Table 1) that not only prevented certain death of untreated animals but even resulted in weight gain. Our presentation of data in terms of total nmoles of enzyme and total units of activity per liver makes it unequivocal that net increases had occurred. Concentration data alone, the form in which activity data is usually provided, would not unequivocally establish that a net change occurred. We have confirmed the conclusion of Weber *et al.* [15] that an increase in assayable activity in units per 10^7 liver cells means an increase in enzyme concentrations. However, our data differ. In our experiments, treatment with insulin during the acute phase of diabetes did not reverse the increase in enzyme activity as it did in the case of the rat experiments by Weber *et al.* [15]. In addition, with our assays we were able to demonstrate that, as treatment proceeded, there was a disparity between total enzyme activity and total enzyme content, i.e. activity did not keep pace with enzyme content. Therefore, with treatment over 10–15 days the specific activity of the enzyme decreased significantly below the control value. The liver cells may have been synthesizing a form of the enzyme that was less active than the enzyme of control animals, which could not be deduced from activity measurements alone. Direct evidence that these possible activity differences between normal and diabetic fructose bisphosphatase are due to specific molecular differences is being sought by comparing carefully characterized, purified enzyme preparations from normal and insulin-treated diabetic animals.

We considered the possibility that alloxan itself may have caused changes in FBPase concentration and specific activity. However, two rabbits did not develop diabetes after administration of alloxan, and enzyme activity and concentration remained normal. In addition, we have shown before that alloxan does not induce liver pyruvate kinase [16], glycerol-3-phosphate dehydrogenase (unpublished observation), or skeletal or cardiac muscle phosphofructokinase [17], or pyruvate kinase [18]. Alloxan diabetes did cause a decrease in the specific activity of liver pyruvate kinase, but insulin therapy quickly returned it to normal. Liver pyruvate kinase had a specific activity of 12.7 units/nmole ± 1.0 (nine animals) in the chow fed animal. The value decreased to 9.6 ± 1.5 (eight anaimals) in the untreated diabetic ($P < 0.005$) but was restored to 11.5 ± 1.8 (four animals) after insulin replacement therapy [16]. Thus, adequate response of the liver to insulin was established, at least with respect to pyruvate kinase. The same groups of animals were used in both the pyruvate kinase and FBPase studies.

Although it appears unlikely that alloxan itself was responsible for the changes in FBPase, this possibility cannot be ruled out at this time. An attempt to use streptozotocin to induce diabetes in rabbits was unsuccessful in our hands.

We cannot claim that perfect diabetes control was achieved by our insulin administration protocol; multiple daily plasma and urine glucose analyses are not possible with rabbits. The administration of single daily doses of insulin, larger than those reported here, was noted to cause hypoglycemia and death in some instances. In contrast, with the dose of lente insulin that we used there were never any clinical signs of hypoglycemia. The rabbits had access to food constantly. Lente insulin injections were given each morning; the time of maximum insulin action could be expected to coincide with the daytime feeding habit of the rabbit. The likelihood of prolonged periods of hypoglycemia was remote.

Normally, newly secreted insulin enters the portal system, and the liver is the first organ to receive the hormone, usually in massive amounts. Insulin injected subcutaneously enters the general circulation, so the liver cannot be the preferred recipient organ. Of course, it continues to receive glucagon directly. Our inability to deliver replacement insulin into the portal system may account for failure of the hepatocyte to normalize its internal enzyme-milieu promptly. Perhaps the achievement of meticulous control of diabetes by multiple daily injections of short-acting insulin would have restored the liver FBPase concentration to normal quickly. Nevertheless, it was unexpected that an amount of daily injected insulin, sufficient to permit weight gain and to restore liver pyruvate kinase activity to normal, was not sufficient to decrease the concentration of liver enzyme.

In summary, fructose bisphosphatase (nmoles) was measured in rabbit livers by a specific radioimmunoassay method in control, alloxan-diabetic, and insulin-treated diabetic rabbits. Enzyme activity measurements were also made. In acutely diabetic animals, enzyme content and activity per liver increased equally (2-fold) over control values. Paradoxically, after 10 days of daily insulin therapy, sufficient to restore general good health and to allow weight gain, enzyme content per liver increased 9-fold, while activity increased only 4-fold over non-diabetic controls. The specific activity decreased from 2.6 units/nmole of enzyme in control animals to 1.5. Thus, large amounts of a less active, cross-reacting form(s) of the enzyme appeared, despite insulin therapy. Even 2 weeks of insulin therapy did not ensure a return to normal of enzyme concentration and specific activity. A possible direct effect of alloxan on the liver is discussed.

Acknowledgements—Supported by grants from the Mayo Foundation, the NIH, the ADA (Minnesota Affiliate), and the JDF.

Table 1. Liver fructose bisphosphatase in alloxan diabetes*

Group	Condition	Animal wt (g)	Liver wt (g)	μmoles 1000 g liver	nmoles liver	units g liver	units liver	units nmole enzyme	N
(1)	Normal	1334 ± 173	60 ± 14	2.7 ± 0.9	157 ± 48	7.1 ± 1.7	413 ± 78	2.6 ± 0.5	10
(2)	Diabetic, untreated	1251 ± 167	49 ± 10 P < 0.05	6.2 ± 1.7 P < 0.001	301 ± 90 P < 0.001	15.6 ± 3.2 P < 0.0001	764 ± 218 P < 0.001	2.6 ± 0.5	13
(3)	Diabetic, treated 2 days	1335 ± 106	86 ± 19	6.8 ± 0.8	575 ± 54	18.0 ± 4.4	1490 ± 101	2.6 ± 0.4	3
(4)	Diabetic, treated 4 days	1535 ± 199	66 ± 5	12.6 ± 1.6	822 ± 118	23.0 ± 1.7	1501 ± 78	1.8 ± 0.3 P < 0.005	4
(5)	Diabetic, treated 8 days	1508 ± 213	77 ± 18	13.4 ± 1.7	1041 ± 340	19.6 ± 3.0	1531 ± 516	1.5 ± 0.2 P < 0.005	4
(6)	Diabetic, treated 10 days	1534 ± 278	76 ± 12	17.1 ± 8.9	1408 ± 928	21.1 ± 9.7	1685 ± 1005	1.3 ± 0.3 P < 0.0005	5
(7)	Diabetic, treated 15 days	1536 ± 124	90 ± 13	14.9 ± 6.8	1342 ± 585	15.4 ± 2.0	1394 ± 323	1.2 ± 0.4	6

* Diabetic animals of group 2 had been given alloxan 48, 72 or 92 hr before they were killed. All treated diabetic animals received alloxan 72 hr before the onset of therapy. At sacrifice plasma glucose of group 2 rabbits was 559 mg/100 ml ± 79. Preceding the start of insulin therapy, plasma glucose for six rabbits of groups 3–6 was 516 ± 50; glucosuria in the remaining ten rabbits was 6.0 g/100 ml ± 3.3. Rabbits were treated with a daily subcutaneous injection (2 units) of Lente insulin.

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