Impaired Regulation of Hepatic Fructose-1,6-Bisphosphatase in the New Zealand Obese Mouse: An Acquired Defect

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Increased hepatic glucose production, a feature of (non–insulin-dependent diabetes mellitus [NIDDM]), is present at an early age in the New Zealand Obese (NZO) mouse and is associated with impaired suppression of the gluconeogenic enzyme, fructose-1,6-bisphosphatase (FBPase). The aim of this study was to further characterize the abnormality in the regulation of hepatic FBPase in NZO mice versus New Zealand Chocolate (NZC) control mice. At 20 weeks of age, NZO mice have elevated FBPase activity (65.3 ± 7.9 *v* 46.7 ± 5.0 μmol/min/mg protein, $P = .07$) and protein levels (31.7 ± 3.1 *v* 22.5 ± 2.8 arbitrary units, $P < .05$), but not mRNA levels (0.18 ± 0.03 *v* 0.16 ± 0.03 arbitrary units). Elevated FBPase activity and protein levels in NZO mice were also shown at 4 to 6 weeks of age, but not in 1-day-old mice, suggesting that the increase occurs between birth and weaning. The K_m of the enzyme was the same in NZO and NZC mice (3.7 ± 0.5 *v* 5.0 ± 0.9 μmol/L, NZO *v* NZC). The regulation of FBPase by the competitive inhibitor, fructose-2,6-bisphosphate ([Fru(2,6)P_2] 5 μmol/L) measured over a range of substrate concentrations (2.5 to 80 μmol/L) was similar between NZO and control mice (K_m in the presence of Fru(2,6)P_2, 10.8 ± 1.9 *v* 13.2 ± 3.3 μmol/L, NZO *v* NZC). It is concluded that increased FBPase activity in the NZO mouse is due to elevated protein levels, and that this appears to be due to a failure of the normal decrease that occurs following birth in control animals.
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INCREASED HEPATIC GLUCOSE production is a characteristic feature of non–insulin-dependent diabetes mellitus (NIDDM) and the major cause of basal hyperglycemia,[1] and is mainly due to increased gluconeogenesis.[2] The biochemical cause of increased gluconeogenesis is not known, but studies in obese NIDDM patients have shown increased conversion of both glycerol and lactate to glucose.[3-5] It has been proposed that the increase in glycerol gluconeogenesis may be due to an increase in the activity of fructose-1,6-bisphosphatase (FBPase).[4]

The New Zealand Obese (NZO) mouse is a polygenic model of NIDDM that has been shown to have hepatic insulin resistance.[6] We have recently demonstrated that the gluconeogenic enzyme FBPase is abnormally regulated in livers of NZO mice. While the activities of two other gluconeogenic enzymes, phosphoenolpyruvate carboxykinase and glucose-6-phosphatase, are appropriately suppressed by the prevailing hyperinsulinemia and hyperglycemia, the activity of FBPase remains inappropriately high.[7] FBPase activity is also elevated in other animal models of insulin resistance and NIDDM.[8-10]

In the gluconeogenic pathway, FBPase catalyzes the conversion of fructose-1,6-bisphosphate to fructose-6-phosphate. Long-term, the level of the enzyme is controlled by insulin, which inhibits, and cAMP, which enhances, gene transcription.[11] Acutely, it is regulated by a metabolite of glucose, fructose-2,6-bisphosphate [Fru(2,6)P_2], which inhibits the activity of FBPase in a competitive manner.[12,13] Levels of Fru(2,6)P_2 are controlled by a bifunctional enzyme, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase, that catalyzes its formation and degradation.[14] The activity of this bifunctional enzyme is regulated by cAMP-dependent protein kinase phosphorylation.[15,16]

The aim of the present study was to investigate the reason for the inappropriately elevated activity of FBPase in the liver of the NZO mouse. We report here that the increased activity of this enzyme is due to elevated protein levels, in the absence of an increase in FBPase mRNA. Elevated FBPase enzyme activity and protein levels were also detected at 4 to 6 weeks of age, but not in 1-day-old mice, suggesting that the defect in the regulation of FBPase may be acquired, not genetic. The response of FBPase to its competitive inhibitor, Fru(2,6)P_2, is normal.

MATERIALS AND METHODS

Materials

Chemical reagents were of analytical grade and were purchased from Sigma Chemical (St Louis, MO). All enzymes were purchased from Boehringer Mannheim (Munich, Germany). γ-^{32}P-dATP was purchased from Bresatec (Adelaide, Australia).

Animals

NZO and New Zealand Chocolate (NZC) mice were obtained from the Walter and Eliza Hall Institute (Parkville, Victoria, Australia). The mice were fed ad libitum on a regular laboratory chow, with water available at all times. On the morning of the study, they were anesthetized with an intraperitoneal injection of pentobarbital sodium (Nembutal; Ceva Chemicals, NSW, Australia). After 15 minutes, a tail vein blood sample was taken for plasma glucose and insulin measurements. Thirty minutes after induction of anesthesia, a laparotomy was performed and the liver rapidly frozen in situ using a clamp previously cooled by immersion in liquid nitrogen. One-day-old mice were killed by cervical dislocation, and livers were quickly removed and frozen in liquid nitrogen. The livers were stored at −70°C for not more than a week until assayed.

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Analytical Procedures

Plasma glucose level was measured using a Yellow Springs glucose analyzer (Yellow Springs Instruments, Yellow Springs, OH), which uses a glucose oxidase method. Plasma insulin was assayed by radioimmunoassay (Pharmacia Diagnostics, Uppsala, Sweden) using a double-antibody technique to separate free insulin from bound insulin.

FBPase Enzyme Assay

Livers were homogenized (1:10 wt/vol) in a buffer containing 50 mmol/L triethanolamine (pH 7.2), 0.1 mmol/L dithiothreitol, and 0.25 mol/L sucrose. The homogenates were centrifuged at 40,000× *g* for 40 minutes (2°C), and the supernatants were used for protein estimation and enzyme activity. FBPase activities at maximal substrate concentrations were assayed by the enzyme-coupled spectrophotometric method of Pontremoli et al[17] by following the reduction of $NADP^+$ to NADPH at 340 nm with a Beckman DU-50 spectrophotometer (Beckman Instruments, Melbourne, Australia).

Protein was determined in supernatants assayed for enzyme activity, with the use of a Bio-Rad microassay protein kit (Richmond, CA). The protein assay is based on the Coomassie blue method using bovine serum albumin as the standard, read at an absorbance of 595 nm.[18]

Kinetic/Inhibition Assays

Livers from overnight fasted mice were homogenized in a medium containing 50 mmol/L triethanolamine, 0.15 mol/L sucrose, 0.1 mol/L dithiothreitol, and 0.1 mol/L NaF. The homogenates were centrifuged at 40,000× *g* for 40 minutes (2°C), and to partially purify the enzyme, FBPase was precipitated from the supernatant by addition of a saturated ammonium sulfate solution (40% to 50% vol/vol). This was centrifuged at 10,000× *g* for 15 minutes (2°C). The pellet containing FBPase was resuspended in a medium containing 50 mmol/L triethanolamine, 0.1 mmol/L dithiothreitol, 50 mmol/L NaF, and 30% (vol/vol) glycerol.

FBPase activity was measured as described earlier, at substrate concentrations ranging from 2.5 to 80 μmol/L in the absence or presence of 5 μmol/L Fru(2,6)P_2.

Western Blotting

Tissue extracts were prepared as described for the enzyme assay. Western blotting was performed on 10% polyacrylamide gels loaded with 5 μg protein. Proteins were transferred to a nitrocellulose membrane, and FBPase was detected using a rabbit anti–mouse liver FBPase serum, which was a kind gift from Dr H. Mizunuma (Akita University, Akita, Japan). Bands were localized on the nitrocellulose membrane using antirabbit antibody conjugated to horseradish peroxidase, and were visualized by the enhanced chemiluminescence method (Amersham, Sydney, Australia). The bands were quantified using a scanning densitometer (Molecular Dynamics, Melbourne, Australia).

Isolation and Quantitation of FBPase mRNA

Liver (0.2 g) was crushed to powder in liquid nitrogen, and total RNA was extracted and purified using the method of Chirgwin et al.[19] RNA (15 μg) was separated on a denaturing agarose gel (1.4%), followed by Northern transfer overnight.[20] Membranes were hybridized to a 21-bp synthetic oligonucleotide probe for FBPase mRNA. The DNA sequence of the oligonucleotide used was TGG-CTT-TGA-TCG-CGG-TGC-AGA, which is complementary to a region in exon 1 of the rat FBPase DNA sequence.[21] The oligonucleotide was labeled by a standard end-labeling protocol[20] using γ-^{32}P-dATP (100 μCi). Hybridization was performed at 50°C in a buffer containing 5× SSC (1× SSC is 0.15 mol/L NaCl plus 0.015 mol/L sodium citrate), 20 mmol/L NaH_2PO_4 (pH 7.0), 7% SDS, 10× Denhardt solution, and 100 μg denatured ssDNA. Autoradiograms were exposed using two intensifying screens for 24 hours at −70°C. FBPase mRNA intensities were determined using a scanning densitometer (Molecular Dynamics, Victoria, Australia). To normalize the data, a murine β_2-microglobulin cDNA probe was used.[22] All data are expressed as the ratio of FBPase mRNA intensity to β_2 -microglobulin mRNA intensity.

Table 1. Characteristic of 4- to 6-Week-Old and 20-Week-Old NZO and NZC Mice

	NZC		NZO	
Characteristic	4-6 Weeks	20 Weeks	4-6 Weeks	20 Weeks
Weight (g)	19.0 ± 0.7	27.8 ± 0.4	25.4 ± 1.0†	55.8 ± 1.5†
Glucose (mmol/L)	18.8 ± 2.4	13.8 ± 0.6	22.1 ± 1.0*	19.8 ± 1.4*
Insulin (pmol/L)	12.4 ± 0.8	73.0 ± 4.0	25.8 ± 4.6*	206.0 ± 27.0†

NOTE. Values are expressed as the mean ± SEM (n = 10).
*P < .01, †P < .001: *v* NZC.

Statistical Analysis

All results are expressed as the mean ±SEM. Statistical significance (P < .05) was determined with the Mann-Whitney nonparametric test for enzyme activities, and the Wilcoxon signed-rank test for Western blotting.

RESULTS

Characteristics of 4- to 6-Week-Old and 20-Week-Old NZO and NZC Mice

At both 4 to 6 and 20 weeks of age, NZO mice were heavier and had significantly elevated plasma glucose and insulin levels compared with age-matched NZC control mice (Table 1).

Inhibition of FBPase by Fru(2,6)P_2

FBPase was prepared as described earlier to minimize the amount of inhibitor and substrate carried over. Samples were then assayed for FBPase activity in the presence or absence of 5 μmol/L Fru(2,6)P_2. The results are presented in Table 2. The K_m of FBPase in NZO or NZC control mice livers was the same in the presence and absence of added inhibitor. As expected, there was a significant increase in K_m in the presence of Fru(2,6)P_2 in both NZO and NZC mice (Table 2). Since Fru(2,6)P_2 is a competitive inhibitor, its addition to the assay mixture did not change the maximal activity, which was higher in NZO mice (Table 2).

Table 2. Maximal Activity (nmol/min/mg protein) and K_m (μmol/L) Values of FBPase From 20-Week-Old NZO and NZC Control Mice in the Absence or Presence of 5 μmol/L Fru(2,6)P_2

	Without Fru(2,6)P_2		With Fru(2,6)P_2	
Mice	Vmax	K_m	Vmax	K_m
NZC	43.2 ± 5.5	5.0 ± 0.9	33.6 ± 6.0	13.2 ± 3.3†
NZO	61.9 ± 6.5*	3.7 ± 0.5	56.6 ± 4.8*	10.8 ± 1.9†

NOTE. Values are expressed as the mean ± SEM (n = 6).
*P < .05 *v* NZC.
†P < .05 *v* without Fru(2,6)P_2.

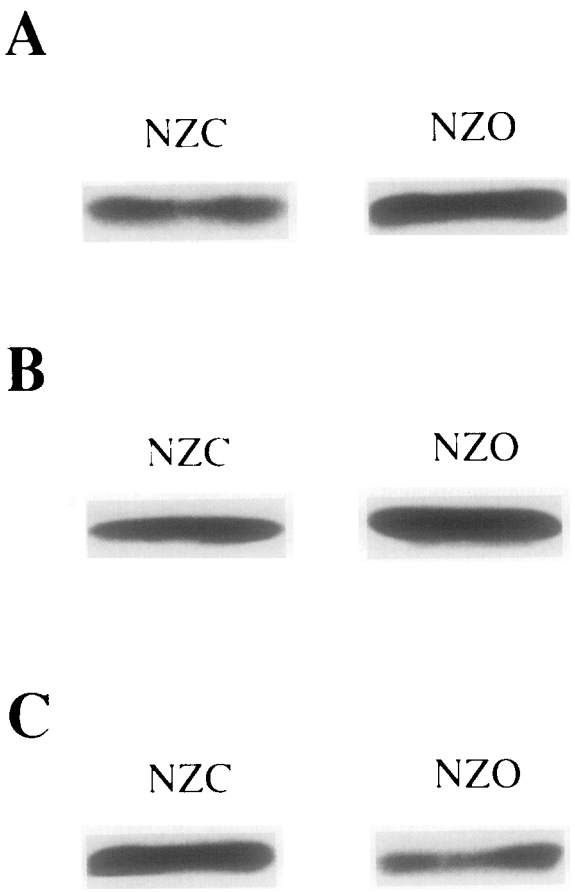

Fig 1. Representative immunoblot of FBPase from NZO and NZC mice. (A) 20-week-old mice, (B) 4- to 6-week-old mice, and (C) 1-day-old mice.

FBPase Activity, Protein, and mRNA levels in 20-Week-Old, 4- to 6-Week-Old, and 1-Day-Old NZO and NZC Mice

At 20 weeks of age, there was a trend for the NZO mouse to have higher FBPase activity, although this did not reach statistical significance (Table 3; $P = .07$). A representative autoradiograph of a FBPase immunoblot measured at 20 weeks of age is shown in Fig 1A, and the mean result from eight mice is shown in Table 3. The molecular weight of the protein band detected was 36,000 daltons, which is consistent with values reported in the literature for mouse liver FBPase.[23] FBPase protein levels were significantly higher in the NZO mouse, and these results correlate well with the maximal enzyme activity (Table 3; $r = .84$, $P < .05$). A representative Northern blot for FBPase of mice at 20 weeks of age is shown in Fig 2, and the mean results are in Table 3. The size of the FBPase mRNA band was approximately 1.4 kb, which agrees well with the value reported in the literature.[11] There was no difference in relative FBPase mRNA levels between NZO and control mice.

At 4 to 6 weeks of age, NZO mice had higher FBPase activity and protein levels (Table 3 and Fig 1B) despite higher plasma insulin and glucose levels (Table 1).

To determine whether the defect in FBPase regulation is congenital, we measured the activity and protein levels of this enzyme in 1-day-old mice. Both the activity and protein levels of FBPase were lower in 1-day-old NZO mice, implying that the defect is acquired (Table 3 and Fig 1C).

DISCUSSION

Increased gluconeogenesis is the driving force for elevated hepatic glucose production and basal hyperglycemia, characteristic of NIDDM,[5] and occurs despite hyperinsulinemia. The biochemical basis for this increased gluconeogenesis is not known. Recent studies have shown that the conversion of glycerol to glucose is elevated in patients with NIDDM.[3,4] Moreover, it was shown that this increase in glycerol gluconeogenesis is due not only to an increase in substrate supply, but also to an increase in the intrahepatic mechanism responsible for conversion of glycerol to glucose.[4] On this basis, Nurjhan et al[4] have postulated an increase in the activity of FBPase in NIDDM. We have previously shown inappropriately high activity of FBPase[7] in the NZO mouse, a polygenic model of NIDDM. The present study was undertaken to further investigate the abnormal regulation of this enzyme in NZO mice and to determine whether it is a possible primary defect in hepatic glucose overproduction.

We have reported that NZO mice have elevated levels of the competitive inhibitor of FBPase, Fru(2,6)P_2.[7] To exclude a defect in the kinetic behavior of FBPase in NZO mice, FBPase activity was measured at substrate concentrations ranging from 2.5 to 80 μmol/L in the absence and presence of 5 μmol/L Fru(2,6)P_2. For this study, mice were fasted overnight and the enzyme was precipitated from the crude homogenate with a saturated ammonium sulfate

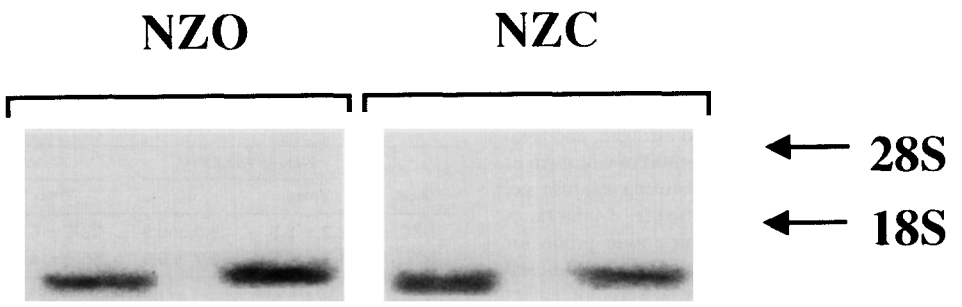

Fig 2. Representative Northern blot of FBPase from 20-week-old NZO and NZC mice.

Table 3. FBPase Activity, Protein, and mRNA Levels in NZO and NZC Control Mice at 1 Day, 4 to 6 Weeks, and 20 Weeks Old

	NZC			NZO		
Parameter	1 Day	4 to 6 Weeks	20 Weeks	1 Day	4 to 6 Weeks	20 Weeks
Activity	98.5 ± 3.5	59.1 ± 4.0	46.7 ± 5.0	78.2 ± 4.4*	76.2 ± 5.8*	65.3 ± 7.9‡
Protein	54.4 ± 4.3	21.8 ± 2.0	22.5 ± 2.8	24.1 ± 3.8*	38.2 ± 2.1†	31.7 ± 3.1*
mRNA	N/A	N/A	0.16 ± 0.03	N/A	N/A	0.18 ± 0.03

NOTE. Results are expressed as the mean ± SEM. Enzyme activity was measured at maximal substrate concentration and expressed as nmol/min/mg protein (n = 8). Protein levels are expressed as arbitrary densitometry units (n = 8), and mRNA levels as the ratio of β_2-microglobulin in arbitrary densitometry units (n = 12).

Abbreviation: N/A, not assayed.

*$P < .01$, †$P < .001$, ‡$P = .07$: *v* NZC.

solution to minimize the amount of substrate and inhibitor present). The results show that there is no difference in K_m in either the absence or the inhibitor. As expected, there was a significant increase in K_m in both NZO and NZC mice with the addition of Fru(2,6)P_2. The maximal activity was higher in the NZO mouse in both the absence and the presence of inhibitor, correlating with results obtained in crude liver homogenates (Table 3). Therefore, the kinetic behavior of FBPase is comparable between NZO and NZC control mice, and suggests that the protein from the NZO mouse is structurally normal.

The cause of the elevated maximal FBPase activity in 20-week-old NZO mice is shown to be due to an increase in the levels of the protein (Fig 1A). To investigate the mechanism for increased FBPase protein, FBPase mRNA level was measured. Table 3 shows that there is no difference in FBPase mRNA between 20-week-old NZO and NZC mice. Thus, the increased protein levels of FBPase cannot be explained by higher levels of mRNA, and may thus be due either to increased stability of the protein or to an increase in the rate of translation.

The same defect in FBPase was also detected in 4- to 6-week-old mice (Table 3 and Fig 1B), consistent with our previous finding of increased hepatic glucose production in NZO mice at this age.[6] To determine whether the increased level of FBPase is congenital, we measured the activity and protein levels in 1-day-old mice and, contrary to what was expected, found that both of these were decreased in NZO mice (Table 3 and Fig 1C). This decrease may be due to the higher maternal plasma glucose levels stimulating fetal insulin secretion, keeping FBPase levels in 1-day-old NZO mice lower as compared with control mice. Thus, the higher FBPase levels in adult NZO mice appear to be due to a failure of the enzyme protein levels to decrease following birth as they do in control lean animals.

It is concluded that adult NZO mice have increased FBPase enzyme activity resulting from elevated protein levels, and that this defect is acquired early in life. The enzyme responds normally to its physiologic competitive inhibitor, Fru(2,6)P_2.

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