Impaired Regulation of Hepatic Fructose-1,6-Bisphosphatase in the New Zealand Obese Mouse Model of NIDDM

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The New Zealand obese mouse, a model of NIDDM, is characterized by hyperglycemia, hyperinsulinemia, and hepatic and peripheral insulin resistance. The aim of this study was to investigate the biochemical basis of hepatic insulin resistance in NZO mice. Glycolytic and gluconeogenic enzyme activities were measured in fed and overnight fasted 19- to 20-wk-old NZO and control New Zealand chocolate mice. The NZO mice were twice as heavy as the NZC mice. The activity of the glycolytic enzymes glucokinase and pyruvate kinase was higher, whereas that of the gluconeogenic enzymes PEPCK and glucose-6-phosphatase was lower in fed and fasted NZO mice. These enzyme changes are consistent with a normal response to the hyperinsulinemia in NZO mice. In contrast, the activity of the third regulated gluconeogenic enzyme, fructose-1,6-bisphosphatase, was similar in fed and fasted NZO and NZC mice despite the higher insulin and glucose levels in the NZO mouse. This enzyme is primarily regulated by the powerful inhibitor fructose-2,6-bisphosphate. The levels of this metabolite were measured and found to be increased in both the fed and fasted states in the NZO mouse, suggesting that the activity of the bifunctional enzyme that regulates the level of inhibitor (6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase) is normally regulated in the NZO mouse. We conclude that most insulin-responsive gluconeogenic and glycolytic enzymes are normally regulated in the NZO mouse, but an abnormality in the regulation of fructose-1,6-bisphosphatase may contribute to the increased hepatic glucose production in these mice. *Diabetes* 42:1731–36, 1993

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Received for publication 22 February 1993 and accepted in revised form 8 July 1993.

NIDDM, non-insulin-dependent diabetes mellitus; HGP, hepatic glucose production; RIA, radioimmunoassay; BSA, bovine serum albumin; DTT, dithiothreitol; cpm, counts per minute.

Hepatic insulin resistance is an important characteristic of NIDDM and is a contributing factor to the elevated plasma glucose levels observed in this disorder (1). Normal or elevated HGP is present in NIDDM despite an increase in fasting insulin and glucose, two factors known to inhibit HGP (2,3). The biochemical basis of this hepatic insulin resistance is not known.

Efforts to identify the defects that cause hepatic insulin resistance have involved, among others, measurement of the activity of key enzymes of the gluconeogenic, glycolytic, or lipogenic pathways (4–8). For example, in the *db/db* mouse, the gluconeogenic enzymes PEPCK, fructose-1,6-bisphosphatase, and glucose-6-phosphatase were shown to be elevated from an early age despite mild hyperinsulinemia (4). By 8 wk of age, when the mice had become more hyperglycemic and hyperinsulinemic, an additional rise occurred in these gluconeogenic enzymes, and the glycolytic enzyme pyruvate kinase also was elevated. At 4 mo of age, the animals became insulin deficient and very hyperglycemic. This was accompanied by an additional increase in the activities of the gluconeogenic enzymes and by a decrease in pyruvate kinase, suggesting that the rise in pyruvate kinase activity was caused by elevated insulin and that insulin, although unable to normalize the activities of the gluconeogenic enzymes, had partially inhibited them. In adult *ob/ob* (8) and KK (6) mice the pattern of liver enzymes is similar to that found in 8-wk-old *db/db* mice.

The NZO mouse is characterized by genetically determined obesity, accompanied by insulin resistance, glucose intolerance, and hyperinsulinemia (9). Unlike many of the other animal models such as the *fa/fa* rat and *ob/ob* and *db/db* mice, it is a polygenic model of NIDDM, because it originated from repeated inbreeding of a mixed mouse colony. The NZO mouse has been shown to have increased HGP at both 4 and 20 wk of age (10).

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This increased HGP is present both basally and under euglycemic-hyperinsulinemic clamp conditions. The aim of this study was to investigate the biochemical basis of this increased HGP in the NZO mouse. This was achieved by measuring the activity of key gluconeogenic and glycolytic enzymes in the fed and overnight fasted states in NZO and control mice. Feeding and fasting was used to study the physiological regulation of these enzymes. We show that in this mouse an hepatic defect can be localized to the regulation of a single gluconeogenic enzyme, fructose-1,6-bisphosphatase.

RESEARCH DESIGN AND METHODS

Chemical reagents were of analytical grade and were purchased from Sigma (St. Louis, MO). All enzymes were purchased from Boehringer Mannheim (Munich, Germany).

NZO and NZC mice (19- to 20-wk-old) were obtained from the Walter and Eliza Hall Institute (Parkville, Victoria, Australia). This mouse line was established in New Zealand in 1948 by selectively inbreeding mice of the same coat color that had arisen from the same colony. Mice with the agouti coat color were inbred for obesity and gave rise to the NZO mice, whereas the tan-colored animals remained lean (NZC) (11). The mice were fed ad libitum on a regular laboratory chow unless fasted in which case they were without food for ~18 h (overnight fasting), with water readily available at all times. On the morning of the study, they were anesthetized with a 60 mg/kg intraperitoneal injection of pentobarbital sodium (Nembutal, Ceva, New South Wales, Australia). After 15 min, a tail-vein blood sample was taken for plasma glucose and insulin measurements. Thirty minutes after the induction of anesthesia, a laparotomy was performed, and the liver rapidly frozen in situ with the use of a clamp previously cooled by immersion in liquid nitrogen. The livers were stored at −70°C for ≤1 wk until assayed.

Analytical procedures. Plasma glucose was measured with the use of a Yellow Springs glucose analyzer (Yellow Springs, OH) that uses a glucose oxidase method. Plasma insulin was assayed by RIA (Pharmacia, Uppsala, Sweden) with the use of a double antibody technique to separate free from bound insulin. Protein was determined in the 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 that uses BSA as the standard read at an absorbance of 595 nm.

Enzyme assays. Enzymes were assayed after homogenization of the livers (1:10 wt/vol) in the appropriate buffers. To assay PEPCK, fructose-1,6-bisphosphatase, and glucokinase, livers were homogenized in a buffer containing 50 mM triethanolamine (pH 7.2) and 0.1 mM DTT with added sucrose (0.25 M) for measurement of fructose-1,6-bisphosphatase and EDTA (0.1 mM) and sucrose (0.25 M) for measurement of PEPCK. The homogenates were centrifuged at 40,000 *g* for 40 min (2°C), and the supernatants were used for protein and for enzyme estimations at maximal substrate concentrations. PEPCK activity was assayed according to the oxaloacetate-$H^{14}CO_3$ exchange method of Chang and Lane (12). Fructose-1,6-bisphosphatase was assayed by the method of Pontremoli et al. (13) and glucokinase according to Salas et al. (14).

Glucose-6-phosphatase enzyme activity was assayed after homogenization of the liver in a 0.25 M sucrose:1 mM EDTA (pH 7.0) solution using ~50 μl/mg of tissue. The homogenate was centrifuged at 13,000 *g* for 3 min, the supernatant was collected, and the enzyme was assayed according to Sein and Maw (15) at maximal substrate concentration.

Pyruvate kinase was assayed after homogenization of the liver (1:10 wt/vol) in a medium containing 20 mM NaK phosphate buffer (pH 7.4), 0.15 M sucrose, 0.1 M KCl, 0.1 M DTT, 1 mM EDTA, and 0.1 M NaF. The homogenate was centrifuged at 40,000 *g* for 40 min (2°C). The L-form of pyruvate kinase was precipitated from the supernatant by the addition of a saturated ammonium sulfate solution (30–40% vol/vol). This was centrifuged at 10,000 *g* for 15 min (2°C). The pellet, containing L-pyruvate kinase, was resuspended in a medium containing 20 mM NaK phosphate buffer (pH 7.4), 0.1 mM DTT, 1 mM EDTA, 50 mM NaF, and 30% vol/vol glycerol. The enzyme assay was performed on this resuspended pellet according to the method of Llorente et al. (16) at maximal substrate concentration.

Isolation and quantitation of PEPCK 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. (17). RNA (15 μg) was either denatured with the use of 1 mM EDTA and 10 mM NaOH and applied to a nylon membrane (Z-probe, Bio-Rad) in a slot-blot apparatus or separated on a denaturing agarose gel (1.4%), followed by Northern blot transfer overnight (18). Membranes were then hybridized to a 1.7-kb *Bam*HI/*Eco*RI fragment of rat genomic PEPCK gene (gift from Drs. M. Magnuson and D.K. Granner, Vanderbilt University), labeled with [^{32}P]dATP by random priming (Promega, Madison, WI) to a specific activity of 10^8 cpm/μg. Autoradiograms were exposed using two intensifying screens for 24 h at −70°C. PEPCK mRNA intensities were determined with the use of a model 2202 Laser Densitometer (LKB, Sweden). To normalize the data, a murine β_2-microglobulin cDNA probe was used. All data were expressed as the ratio of PEPCK mRNA intensity to β_2-microglobulin mRNA intensity.

Fructose-2,6-bisphosphate assay. Fructose-2,6-bisphosphate was measured in NaOH neutralized extracts by use of the coupled spectrophotometric assay described by Van Schaftingen et al. (19).

cAMP assay. Hepatic cAMP was assayed by use of the Amersham [^{3}H]cAMP assay system (Amersham International, Amersham, UK). Livers were homogenized in 4 mM EDTA (pH 7.5), and the homogenates placed in a boiling water bath for several minutes to coagulate the proteins. The samples were centrifuged and cAMP assayed in the supernatants.

Statistical analysis. All results are means ± SE. Statistical significance ($P < 0.05$) was determined by use of the Mann-Whitney test.

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Table 1
Characteristics of fed and overnight fasted NZO and NZC mice

	NZC fed mice	NZO fed mice	NZC fasted mice	NZO fasted mice
Weight (g)	27.2 ± 0.2	56.1 ± 1.3*	25.2 ± 0.8†	49.2 ± 1.9*†
Glucose (mM)	14.1 ± 0.6	20.7 ± 1.5‡	7.4 ± 0.5§	13.1 ± 1.2*†
Insulin (pM)	76 ± 5	222 ± 30*	32 ± 3§	75 ± 12†‡

Data are means ± SE; n = 10.
*$P < 0.001$ compared with NZC mice.
†$P < 0.001$ compared with corresponding fed mice.
‡$P < 0.01$ compared with NZC mice.
§$P < 0.0001$ compared with corresponding fed mice.

RESULTS

Characteristics of the animals. The mice used in this study were all between 19 and 20 wk of age. The NZO mice were significantly heavier in both the fed and fasted states compared with the control mice (Table 1). As shown previously (9), NZO mice were hyperglycemic and hyperinsulinemic in both the fed and fasted states compared with NZC mice (Table 1). As expected, fasted mice had lower glucose and insulin levels compared with their corresponding fed littermates.

Glycolytic enzymes. Glucokinase and pyruvate kinase enzyme levels for fed and fasted NZO and NZC control mice are shown in Table 2. Fasting was associated with a decrease in glycolytic enzyme levels in both NZO and NZC mice, although this did not reach statistical significance for pyruvate kinase. In both the fed and fasted states, glucokinase and pyruvate kinase enzyme activities were higher in NZO mice compared with the control mice.

Gluconeogenic enzymes. Table 2 shows that the activity of PEPCK, the first committed enzyme of the gluconeogenic pathway, was lower in both the fed and fasted states in the NZO mice compared with the NZC mice. To confirm the enzyme activity results, PEPCK mRNA was determined (Fig. 1). As with activity, PEPCK mRNA levels rose in both NZO and NZC mice after overnight fasting, and mRNA was lower in the NZO compared with the NZC mice in both the fed and fasted states. These changes suggest an appropriate response of PEPCK to the higher insulin levels in the NZO mice.

The last enzyme in the gluconeogenic pathway is glucose-6-phosphatase. Fasting resulted in the expected increase in activity of this enzyme in both NZO and NZC mice. Like for PEPCK activity, this enzyme was lower in the NZO compared with NZC mice in both the fed and fasted states (Table 2).

The central regulated step in gluconeogenesis is the conversion of fructose-1,6-bisphosphate to fructose-6-phosphate, which is catalyzed by the enzyme fructose-1,6-bisphosphatase. Table 2 shows that, as with the other gluconeogenic enzymes, maximal fructose-1,6-bisphosphatase activity increased in response to fasting. However, unlike the other gluconeogenic enzymes, the activity per milligram of liver protein in the NZO mice was not lower than in the NZC.

Regulation of the inhibitor of fructose-1,6-bisphosphatase. An important regulator of gluconeogenesis is the level of a metabolite of glucose, fructose-2,6-bisphosphate. This compound activates 6-phosphofructo-1-kinase and inhibits fructose-1,6-bisphosphatase, thus resulting in a stimulation of glycolysis and an inhibition of gluconeogenesis. The levels of fructose-2,6-bisphosphate are determined by the availability of glucose and by the activity of a complex enzyme that both produces and degrades the compound, fructose-2,6-bisphosphatase/6-phosphofructo-2-kinase. This latter enzyme is in turn regulated by cAMP. Phosphorylation of the bifunctional enzyme by protein kinase A activates the bisphosphatase and inhibits the kinase activity. Thus, fructose-2,6-bisphosphate normally increases with feeding and

Table 2
Glycolytic and gluconeogenic enzyme levels in fed and overnight fasted NZO and NZC mice

	NZC fed mice	NZO fed mice	NZC fasted mice	NZO fasted mice
Glycolytic enzymes				
Glucokinase	8.3 ± 1.0	13.1 ± 1.7*	5.7 ± 0.4†	8.9 ± 1.0*†
Pyruvate kinase	82.4 ± 11.8	163.4 ± 28.7*	67.5 ± 7.9	123.7 ± 17.6*
Gluconeogenic enzymes				
PEPCK	19.0 ± 1.0	12.0 ± 2.0*	42.0 ± 1.0‡	20.0 ± 1.0*
Fructose-1,6-bisphosphatase	58.1 ± 4.4	68.9 ± 6.9	85.3 ± 9.2†	98.6 ± 10.1†
Glucose-6-phosphatase	128.7 ± 6.0	102.4 ± 5.5§	152.6 ± 2.4†	120.1 ± 5.1†§

Data are means ± SE; n = 6–8.
*$P < 0.05$ compared with NZC mice.
†$P < 0.05$ compared with corresponding fed mice.
‡$P < 0.001$ compared with corresponding fed mice.
§$P < 0.01$ compared with NZC mice.

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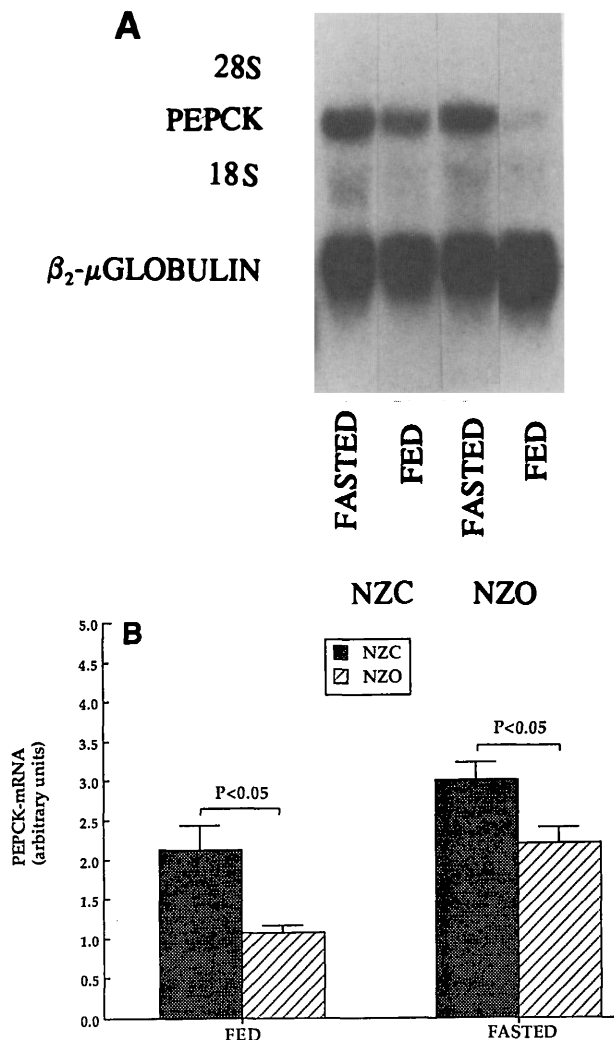

FIG. 1. *A*: Northern blot of PEPCK mRNA isolated from fed and overnight fasted NZO and NZC mice. Liver mRNA was prepared as described in METHODS and probed with PEPCK and β_2-microglobulin probes. *B*: PEPCK mRNA from fed and overnight fasted NZO and NZC mice. Autoradiograms from slot-blots were quantitated by scanning densitometry. Data are means ± SE (n = 5–8) of the ratio of PEPCK to β_2-microglobulin.

decreases with fasting. Figure 2 shows that fructose-2,6-bisphosphate is higher in the fed state in both strains of mice, but as with the activity of the glycolytic enzymes, it is higher in the NZO mouse. To determine whether the bifunctional enzyme is normally regulated in the NZO mouse, we calculated the difference between fasting and feeding for the obese and lean mice. The difference in NZO mice was 0.700 ± 0.169 nmol/g liver and in NZC mice it was 0.706 ± 0.174 nmol/g liver, suggesting that the regulation of the activity of the bifunctional enzyme is normal in the NZO mouse.

Hepatic cAMP levels. Hepatic cAMP levels are represented in Fig. 3. Overnight fasting caused a significant increase in cAMP in both NZO and NZC mice; however, no difference was noted between NZO and control mice in either the fed or fasted states.

DISCUSSION

Hepatic insulin resistance is an important contributor to fasting hyperglycemia in NIDDM patients (1,20). However, the biochemical basis for this resistance is not understood. Studies in humans have described defects in insulin receptor binding (21) and postbinding events (22), and several studies have documented increases in the activity of gluconeogenic enzymes in a variety of hyperinsulinemic animal models of NIDDM (4–8).

We have shown previously that hepatic insulin resistance is an early feature of the NZO mouse (10), being present at both 20 and 4 wk of age. In this study we show, in the NZO mouse, that hepatic resistance to insulin is not global, but rather confined to a single gluconeogenic enzyme with all other measured steps in the glycolytic and gluconeogenic pathways being normal for the prevailing insulin and glucose levels. Thus, the activity of PEPCK and glucose-6-phosphatase are decreased, whereas that of glucokinase and pyruvate kinase are increased in the NZO mice compared with that in the NZC mice. Furthermore, fructose-2,6-bisphosphate, a metabolite of glucose that provides a switch mechanism between gluconeogenesis and glycolysis (23), was appropriately increased in the NZO mice (Fig. 2), suggesting that the activity of the bifunctional enzyme that regulates the level of this metabolite is also normally controlled in the NZO mice.

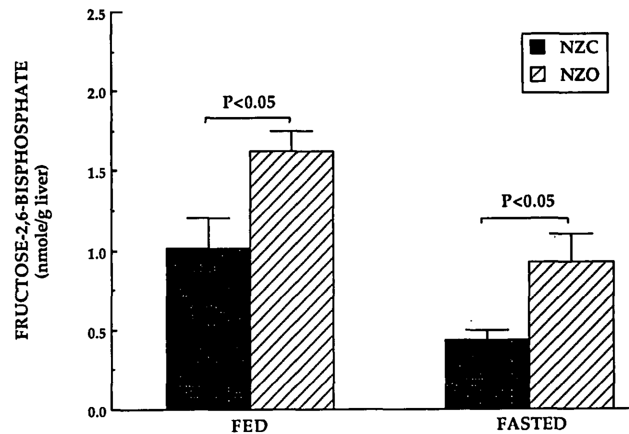

FIG. 2. Fructose-2,6-bisphosphate levels in fed and overnight fasted NZO and NZC mice. Data are means ± SE (n = 8).

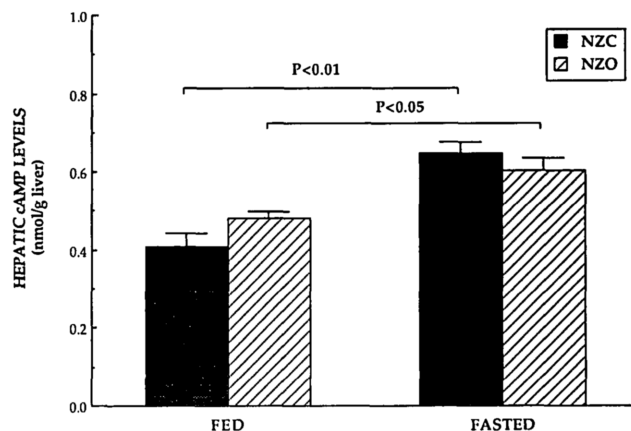

FIG. 3. Hepatic cAMP levels in NZO and NZC control mice. Data are means ± SE (n = 5).

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The most important finding of this study is an apparent defect in the inhibition of fructose-1,6-bisphosphatase in the NZO mouse. Unlike the other two regulatory gluconeogenic enzymes measured, no difference was noted in the maximal enzyme activity of fructose-1,6-bisphosphatase in fed and overnight fasted NZO and NZC mice. Fructose-1,6-bisphosphatase was measured at maximal substrate concentration, thus the activity reflects the protein content. Table 2 shows that total activity of fructose-1,6-bisphosphatase is modulated appropriately by fasting or feeding, suggesting that changes in insulin and glucose within the physiological range are able to modulate protein levels. Thus, the hyperinsulinemia and hyperglycemia in the NZO mouse should have caused a lower level in fructose-1,6-bisphosphatase activity, in a similar way to that demonstrated for PEPCK and glucose-6-phosphatase. This can be appreciated best by comparing the insulin, glucose, and enzyme activities of fed NZC and fasted NZO animals (Tables 1 and 2). No difference exists between these two groups in insulin and glucose, but fructose-1,6-bisphosphatase activity is approximately twice as high in the NZO livers (58 ± 4.4 vs. 98.6 ± 10.1 $nmol \cdot min^{-1} \cdot mg\ protein^{-1}$, $P < 0.05$, $n = 9$).

The pattern of gluconeogenic enzymes reported here for the NZO mouse is different from that reported previously in other animal models of NIDDM such as *ob/ob* and *db/db* mice. This difference could be caused by a different etiology of the syndromes in different models. For example, *fa/fa* rats and *ob/ob* and *db/db* mice have single gene mutations as the cause of their syndromes, whereas the NZO mouse is a polygenic model having been produced by repeated inbreeding to select for obesity.

Hepatic cAMP levels were measured in NZO and control mice to determine whether changes in the levels of this cyclic nucleotide could explain the observed changes in the activity of PEPCK and fructose-1,6-bisphosphatase, because it is known that cAMP increases gene transcription of both these enzymes. Figure 3 shows, as expected, that fasting caused a significant increase in cAMP. More importantly, no difference was noted in cAMP levels between NZO and NZC mice in either the fed or fasted states. This suggests that the lower level of PEPCK is not caused by low cAMP levels but is the direct result of elevated insulin levels. Furthermore, the impaired suppression of fructose-1,6-bisphosphatase in the NZO mouse cannot be explained by increased cAMP levels.

An inappropriately active fructose-1,6-bisphosphatase could result in increased conversion of glycerol to glucose because this precursor enters the pathway at this level. Although it is not possible to extrapolate the findings reported here in the NZO mouse to human NIDDM, two recent reports have documented increased gluconeogenesis from glycerol in NIDDM patients (24,25). In both studies evidence was presented that this increase was at least partly caused by an increase in the intracellular conversion of glycerol to glucose and not only by increased glycerol availability. Nurjhan et al. (24) have speculated that this could be the result of increased activity of fructose-1,6-bisphosphatase.

In conclusion, we have shown, in the NZO mouse, that there is impaired regulation of fructose-1,6-bisphosphatase. This defect could contribute to the increased HGP and hyperglycemia observed in this mouse model of NIDDM.

ACKNOWLEDGMENTS

This work was supported by a program grant from the National Health and Medical Research Council of Australia and from a grant-in-aid from Apex/Diabetes Australia Research and Education Grant. J.P. was a Wellcome Australia Senior Research Fellow. J.D.Z. is supported by a Keir Fellowship of the Royal Melbourne Hospital.

We thank Sue Fabris for excellent technical assistance. We also thank Keith Royston for providing the murine β_2-microglobulin cDNA probe.

This work fulfills portions of the PhD thesis requirements for S.A.

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