Ketosis Resistance in the Male Offspring of Protein-Malnourished Rat Dams

S.E. Ozanne, C.L. Wang, C.J. Petry, J.M. Smith, and C.N. Hales

Plasma β-hydroxybutyrate concentrations were measured in the offspring of rats that were fed either a control (20% protein) diet or low-protein (8% protein) diet during pregnancy and lactation. Low-protein offspring had significantly lower plasma β-hydroxybutyrate compared with controls in the fed state ($P < .04$) and after fasting for 24 hours ($P < .001$) and 48 hours ($P < .04$). There were no differences in blood glucose, acetoacetate, plasma glucagon, cholesterol, or glycerol between control and low-protein offspring. However, plasma nonesterified fatty acids (NEFAs) were significantly higher in low-protein offspring in the fed state ($P < .05$). In contrast, plasma triglycerides and insulin were significantly lower in low-protein offspring compared with controls when fed ($P < .001$) and after a 24-hour fast ($P < .001$). These results suggest that poor maternal and early postnatal nutrition can have long-term effects on ketone body metabolism in the offspring during adulthood. This apparent ketosis resistance is similar to that observed in some forms of human diabetes.

A GROWING NUMBER of studies have reported a relationship between poor fetal and neonatal growth and the subsequent development of non–insulin-dependent diabetes mellitus (NIDDM) in adulthood.[1] These observations led to the proposal of the thrifty phenotype hypothesis.[2] This hypothesis suggests that during times of maternal nutritional deprivation, the growing fetus uses a number of strategies to ensure survival. Firstly, it is proposed that the fetus protects the growth of the brain at the expense of other organs such as the liver and pancreas. Secondly, it is suggested that permanent metabolic changes occur in tissues such as the liver that are advantageous to survival in conditions of poor postnatal nutrition. However, if the offspring are born into conditions of good/excessive nutrition, then this may conflict with the earlier programing and NIDDM may occur.

Many aspects of the thrifty phenotype have been tested and subsequently supported by an animal model of early growth retardation. In this model, rats are fed either a low-protein (8%) diet or a control (20% protein) diet during pregnancy and lactation.[3] All offspring are then weaned onto a control (20% protein) diet.[3] Low-protein offspring have a lower body weight than controls throughout life. Consistent with proposals in the thrifty phenotype hypothesis, low-protein offspring exhibit selective changes in organ growth, with brain growth being protected.[3] In addition, permanent changes in the metabolism of the liver,[4] muscle,[5] and fat[6,7] were observed. Although low-protein offspring showed better glucose tolerance than controls in young adult life,[8] they exhibited a greater time-dependent loss of glucose tolerance such that at 15 months of age they were significantly less glucose-tolerant than controls.[8]

There are a number of studies suggesting that some people with NIDDM[9] and malnutrition-related diabetes are relatively unable to produce the ketone body β-hydroxybutyrate.[10] In particular, individuals with fibrocalculous pancreatic diabetes (a subtype of malnutrition-related diabetes) do not become ketotic even in adverse conditions.[11] However, the precise molecular basis of this ketosis resistance is not understood. It could occur through a diminished supply of nonesterified fatty acids (NEFAs) from adipose tissue and/or a reduced hepatic ketogenic response. There have been suggestions that the latter may be, in part, a result of glucagon resistance, glucagon being a key regulator of ketone production by the liver.[12] It has been shown previously that both chronically malnourished rats[13] and the offspring of rats fed a low-protein diet during pregnancy and lactation are glucagon-resistant.[4] In the low-protein offspring, we have shown that this glucagon resistance is related to a fivefold reduction in the number of glucagon receptors present on the hepatic plasma membranes.[4] Thus, the aim of the present study was to determine the ability of the offspring of rats that were fed a low-protein diet during pregnancy and lactation to produce ketone bodies during various periods of starvation.

MATERIALS AND METHODS

Materials

Analytic-grade biochemicals were obtained from Sigma Chemical or BDH Biochemicals (both in Poole, Dorset, UK) unless stated otherwise. Linco rat insulin and glucagon radioimmunoassay kits were purchased from Biogenesis (Poole, Dorset, UK).

Animals

All procedures involving animals were performed according to the British Home Office Animals (Scientific Procedures) Act of 1986. Virgin female Wistar rats (initial weight, 240 to 260 g) used for the study were housed individually and maintained at 22°C on a 12-hour light/dark cycle. They were mated, and day 0 of gestation was determined as the day the vaginal plug was expelled. The rats were fed a diet containing 20% protein or an isocalorific diet containing 8% protein throughout pregnancy and lactation. The composition and source of the diets were as described by Snoeck et al.[14]

Spontaneous delivery occurred on day 22 of pregnancy, after which, at age 3 days, the litters were reduced randomly to eight pups, thus ensuring a standard litter size per mother. At 21 days of age, all pups were weaned onto a 20% protein diet. For simplicity, the two groups of offspring are termed "control" and "low-protein"; however, it is emphasized that only the mothers undergo dietary manipulation.

Blood Collection

Three-month-old male rats were studied in the fed state and after starvation for 24 and 48 hours. Blood was collected from the tail vein

From the Department of Clinical Biochemistry, Addenbrooke's Hospital, Cambridge; and the Department of Clinical Biochemistry, Selly Oak Hospital, Birmingham, UK.

Submitted September 29, 1997; accepted June 15, 1998.

Supported by the Medical Research Council, the Parthenon Trust, and the British Diabetic Association.

Address reprint requests to S.E. Ozanne, PhD, Department of Clinical Biochemistry (Level 4), Addenbrooke's Hospital, Hills Road, Cambridge, UK CB2 2QR.

0026-0495/98/4712-0004$03.00/0

Table 1. Circulating Insulin and Glucagon Concentrations in Control and Low-Protein Offspring When Fed and Fasted for 24 and 48 Hours

	Fed		24-Hour Fasted		48-Hour Fasted	
Parameter	Control	Low-Protein	Control	Low-Protein	Control	Low-Protein
Glucagon (pmol/L)	18 (16-19)	19 (18-21)	11 (10-12)	12 (11-13)	13 (12-14)	11 (11-12)
Insulin (pmol/L)	898 (765-1,053)	545 (462-642)*	299 (241-370)	164 (132-250)*	109 (58-208)	134 (83-154)

NOTE. Results are the geometric mean (95% confidence interval); n = 16 per group.
*$P < .001$ *v* control.

into heparinized tubes. Red blood cells were removed by centrifugation, and the plasma was collected and stored at −70°C prior to analysis.

Assay Procedures

Blood glucose was determined using a Hemocue Glucose Analyzer (Hemocue, Sheffield, UK). Plasma glucagon and insulin levels were measured by radioimmunoassay according to the manufacturer's protocol. β-Hydroxybutyrate, total cholesterol, free glycerol, and triacylglycerol levels were measured using kits purchased from Sigma Chemical (Poole, Dorset, UK). Acetoacetate was determined in perchloric acid extracts of whole blood using a fluorimetric assay.[15]

Statistical Analysis

All statistical analyses were performed using the Statistica for Mac (version 4.1) software package (Statsoft, Letchworth, Herts, UK). Student's *t* test and two-way ANOVA were used to analyze the data with early diet and length of starvation as independent variables. When appropriate, the data were logarithmically transformed before analysis to enable the appropriate use of parametric statistical tests. Results are expressed as the mean ± SEM or geometric mean with 95% confidence interval in cases for which the data were log-transformed.

RESULTS

Blood Glucose

Blood glucose concentrations were significantly ($P < .001$) lower after a 24-hour fast (4.9 ± 0.1 and 5.2 ± 0.1 mmol/L in control and low-protein offspring, respectively) compared with the fed state (6.2 ± 0.2 and 5.9 ± 0.1 mmol/L for control and low-protein, respectively). Blood glucose was even lower ($P < .001$) after a 48-hour fast (4.5 ± 0.2 for both control and low-protein offspring). However, there were no significant differences in blood glucose concentrations between control and low-protein animals at any time point during the study ($P < .05$).

Plasma Insulin and Glucagon

Plasma glucagon concentrations decreased after a 24-hour starvation period in both study groups ($P < .001$). However, continued starvation for another 24 hours had no futher effect on plasma glucagon (Table 1). There were no significant differences between control and low-protein offspring at any study point. Plasma insulin concentrations decreased with starvation in both experimental groups ($P < .001$ for the effect of starvation). However, plasma insulin was significantly lower in the low-protein group compared with controls when fed ($P < .001$) and after a 24-hour fast ($P < .001$). After 48 hours of starvation, there was no significant difference in plasma insulin levels between the two groups.

β-Hydroxybutyrate, Acetoacetate, and Plasma NEFAs

Plasma β-hydroxybutyrate and blood acetoacetate concentrations increased progressively ($P < .001$ for the effect of increasing starvation on each ketone body) with extended periods of starvation in both control and low-protein offspring (Fig 1A and B). However, at each study point (fed, 24-hour starvation, and 48-hour starvation), low-protein offspring had significantly lower plasma β-hydroxybutyrate compared with controls ($P < .001$ for the effect of the low-protein diet; Fig 1A). In contrast, there were no differences in acetoacetate concentrations between the groups at any time point (Fig 1B). In addition, low-protein offspring had a significantly ($P < .05$) elevated plasma NEFA concentration compared with controls (450 ± 54 *v* 325 ± 29 μmol/L) in the fed state (Fig 2). Low-protein offspring also tended to have higher plasma NEFA after 24 hours of starvation; however, this did not reach statistical significance in the number of animals studied (640 ± 61 *v* 510 ± 39 μmol/L, $P = .079$). There were no differences in

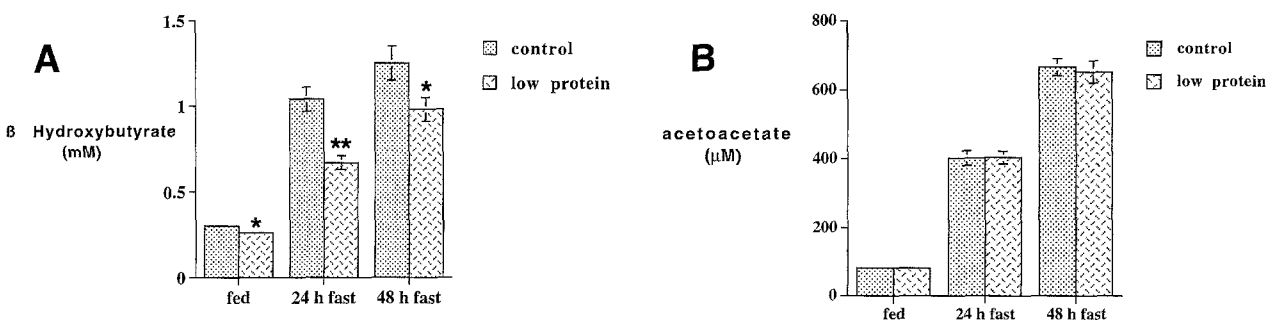

Fig 1. (A) Circulating β-hydroxybutyrate concentrations in control and low-protein offspring in the fed and state and after fasting for 24 and 48 hours. Results are the mean ± SEM (n = 16 per group). *$P < .05$ *v* control. **$P < .001$ *v* control. (B) Circulating acetoacetate concentrations in control and low-protein offspring in the fed state and after fasting for 24 and 48 hours. Results are the mean ± SEM (n = 12 per control group and n = 11 per low-protein group).

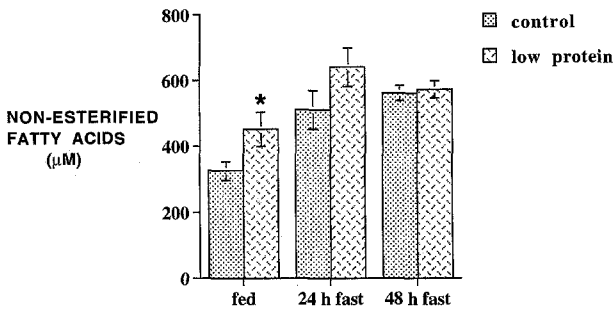

Fig 2. Circulating NEFA concentrations in control and low-protein offspring in the fed state and after fasting for 24 and 48 hours. Results are the mean ± SEM (n = 16 per group). *$P < .05$ v control.

plasma NEFA after 48 hours of starvation (561 ± 23 and 572 ± 26 μmol/L for control and low-protein, respectively).

Cholesterol, Triglyceride, and Free Glycerol

There were no differences in plasma cholesterol or glycerol concentrations between the two groups at any study point ($P > .05$; Table 2). However, plasma cholesterol decreased progressively with increasing starvation in both groups ($P < .001$), whereas starvation had no effect on plasma glycerol after 24 hours but resulted in a significant ($P < .001$) decrease in glycerol after 48 hours. The plasma triglyceride concentration was significantly lower in low-protein offspring compared with controls in the fed state ($P < .001$) and after 24 hours of starvation ($P < .001$; Table 2). After 48 hours of starvation, plasma triglyceride also tended to be lower in low-protein offspring ($P = .093$); however, this did not reach statistical significance in the number of animals studied. Plasma triglycerides decreased with an increasing starvation period in control animals ($P < .001$). In the low-protein group, triglycerides decreased after 24 hours of starvation ($P < .001$), but no further decrease was observed when the starvation period was increased to 48 hours ($P > .05$).

DISCUSSION

The main aim of the present study was to investigate the possibility that the offspring of protein-malnourished rats have long-term alterations in their ability to produce/metabolize ketone bodies. We have been using this model as a test of the thrifty phenotype hypothesis of the etiology of NIDDM. Ketosis resistance (in terms of the level of the major ketone body, β-hydroxybutyrate) is a feature of certain forms of NIDDM and malnutrition-related diabetes.[9,10] The offspring of rats fed a low-protein diet during pregnancy and lactation are permanently growth-retarded and show a greater time-dependent loss of glucose tolerance. They also demonstrate other, often unexplained features of human NIDDM. For example, at 3 months of age, male low-protein offspring exhibit an altered hepatic response to insulin.[4] The hormone causes an initial paradoxical increase in hepatic glucose output before the expected inhibition of glucagon-stimulated hepatic glucose output. A similar paradoxical effect has also been reported in individuals with NIDDM[16] and in Aborigines (who have a high risk of developing NIDDM).[17] In these studies, administration of glucose led to an initial increase in hepatic glucose output before a decrease occurred. The mechanism(s) of this effect in humans and in the low-protein animal model is not clear.

In the current study, 3-month-old male rats from both experimental groups produced more ketone bodies (both β-hydroxybutyrate and acetoacetate) with increasing starvation. However, low-protein offspring had significantly lower plasma β-hydroxybutyrate concentrations in the fed state and after a 24-hour and 48-hour starvation period. This observation may be due to a relative increase in the utilization and/or a decrease in the production of β-hydroxybutyrate by low-protein offspring. At this stage, neither possibility can be excluded. A relative increase in ketone body utilization may occur if the whole body composition was altered in low-protein offspring. However, no such data are currently available. In contrast to the differences observed with β-hydroxybutyrate, there were no differences in acetoacetate concentrations between the two groups. This may suggest that there is a specific alteration in the ability to produce β-hydroxybutyrate (the major ketone body). It is not clear if this specific effect on β-hydroxybutyrate characterizes human malnutrition-related diabetes, as data on acetoacetate were not presented in these subjects.[11]

A relative inability to produce the ketone body β-hydroxybutyrate could be due to a diminished supply of NEFAs from adipose tissue or a diminished ability of the liver to metabolize NEFAs to ketone bodies. However, in the current study, low-protein offspring had higher plasma NEFA concentrations compared with controls when fed and after a 24-hour starvation. In addition, if there was a reduced supply of NEFAs, one would also expect acetoacetate concentrations to be lower. This suggests that the reduced concentration of β-hydroxybutyrate in these animals does not result from a lack of substrates for its production. The mechanism of the observed elevation of NEFA in the low-protein offspring is not clear. It is possible that hepatic uptake of NEFAs is impaired in low-protein offspring. Indeed, this has been suggested as a mechanism to explain an observed increase in peripheral concentrations of NEFAs in

Table 2. Circulating Triglyceride, Cholesterol, and Glycerol Concentrations in Control and Low-Protein Offspring When Fed and Fasted for 24 and 48 Hours

	Fed		24-Hour Fasted		48-Hour Fasted	
Parameter	Control	Low-Protein	Control	Low-Protein	Control	Low-Protein
Triglycerides (mmol/L)	2.71 ± 0.16	1.92 ± 0.13*	1.08 ± 0.08	0.61 ± 0.06*	0.68 ± 0.04	0.60 ± 0.04
Cholesterol (mmol/L)	2.82 ± 0.09	2.59 ± 0.08	2.02 ± 0.07	2.02 ± 0.08*	1.82 ± 0.14	1.87 ± 0.08
Glycerol (mmol/L)	0.51 ± 0.02	0.53 ± 0.02	0.59 ± 0.02	0.57 ± 0.02	0.41 ± 0.02	0.37 ± 0.01

NOTE. Results are the mean ± SEM (n = 16 per group).
*$P < .001$ v control.

some NIDDM patients during medium-chain triglyceride feeding studies.[11] Alternatively, the increased levels of NEFAs could result from increased release by adipocytes in low-protein offspring. Consistent with this possibility is the observation that in the fed state and after a 24-hour starvation, low-protein offspring have lower plasma insulin but similar plasma glucagon levels compared with controls. An increased glucagon to insulin ratio is thought to favor NEFA release from adipocytes.[18]

Following uptake by the liver, NEFAs may be esterified to triglycerides and secreted in the form of very–low-density lipoprotein, or they may enter the oxidative pathway and ketogenesis. Thus, another possible mechanism to explain the decreased levels of β-hydroxybutyrate would be that upon uptake by the liver in low-protein animals, a greater relative amount of NEFAs were reesterified than oxidized. This possibility could be addressed in the future by measuring liver triglyceride levels in the two animal groups.

Other possible explanations for the ketosis resistance include a defect in the ability of a reduced insulin concentration and/or increased glucagon concentration to stimulate ketogenesis. It is thought that the major effect of insulin on ketogenesis during starvation occurs via regulation of the NEFA supply by its action on hormone-sensitive lipase in adipocytes.[19] Under normal circumstances, insulin levels during starvation decrease, with the result that hormone-sensitive lipase becomes more active (insulin being an inhibitor of hormone-sensitive lipase). As described already, the decreased concentration of β-hydroxybutyrate in low-protein offspring occurs in the presence of increased NEFA levels. Thus, altered regulation of hormone-sensitive lipase does not seem to play a role in the development of ketosis resistance in this instance.

Glucagon is thought to have a direct hepatic effect to stimulate ketogenesis.[20] Plasma glucagon levels decreased after 24 hours of starvation. The mechanism by which this occurs is not clear, but similar observations have been reported.[21] We have shown previously that the liver of low-protein offspring is resistant to the action of glucagon to stimulate glucose transport, and this is related to a reduction in the number of glucagon receptors in hepatic membranes.[4] In addition, marked alterations of adenylate cyclase activity have been observed in liver plasma membranes of NIDDM patients in response to hyperglucagonemia.[22] Thus, glucagon resistance may contribute to the observed ketosis resistance but, again, would not fully explain why acetoacetate levels are similar in the two groups.

Another possible mechanism by which a change in the β-hydroxybutyrate to acetoacetate ratio might occur is via a change in the redox state of the mitochondria. Acetoacetate is converted to β-hydroxybutyrate by the enzyme β-hydroxybutyrate dehydrogenase in the presence of NADH. Thus, a change in the ratio of NADH to NAD could affect the rate of this reaction. However, if such an alteration was the sole underlying mechanism in the current study, then it might be expected that the lower β-hydroxybutyrate would be accompanied by an elevated acetoacetate.

In summary, the offspring of rats fed a low-protein diet during pregnancy and lactation have lower plasma levels of the ketone body β-hydroxybutyrate than control offspring. The exact biochemical basis for these observations is not clear. Glucagon resistance may contribute to this finding; however, other possibilities such as a change in the expression of enzymes involved in β-hydroxybutyrate production or a change in the utilization of β-hydroxybutyrate must also be addressed. However, regardless of the mechanism, this observation is another example of a feature observed in certain types of human diabetes that is also found in low-protein rat offspring. Therefore, this rat model continues to be a useful tool for the study of biochemical mechanisms underlying human forms of glucose intolerance and NIDDM.

ACKNOWLEDGMENT

We thank D. Hutt, A. Flack, A. Wayman, and L. Smith of the Dunn Nutritional Laboratory Animal Unit for invaluable assistance. We are also grateful to T. Elsey of the National Health Service Trust, Clinical Biochemistry Department, Addenbrooke's Hospital, Cambridge, for help and advice with the β-hydroxybutyrate measurements, and to R.A. Round of the NHS Trust, Department of Clinical Biochemistry, Selly Oak Hospital, Birmingham, for performing the acetoacetate assays.

REFERENCES

1. Philips DIW, Hales CN: The intrauterine environment and susceptibility to non–insulin dependent diabetes and the insulin resistance syndrome, in Marshall SM, Home PD, Rizza RA (eds): The Diabetes Annual, vol 10. Amsterdam, The Netherlands, Elsevier, 1996, pp 1-13

2. Hales CN, Barker DJP: Type 2 (non–insulin-dependent) diabetes mellitus: The thrifty phenotype hypothesis. Diabetologia 35:595-601, 1992

3. Desai M, Crowther NJ, Lucas A, et al: Organ-selective growth in the offspring of protein restricted mothers. Br J Nutr 76:591-603, 1996

4. Ozanne SE, Smith GD, Tikerpae J, et al: Altered regulation of hepatic glucose output in the male offspring of protein malnourished rat dams. Am J Physiol 270:E559-E564, 1996 (suppl)

5. Ozanne SE, Wang CL, Coleman N, et al: Altered muscle insulin sensitivity in the male offspring of protein malnourished rats. Am J Physiol 271:E1128-E1134, 1996 (suppl)

6. Ozanne SE, Nave BT, Wang CL, et al: Poor fetal nutrition causes long term changes in expression of insulin signalling components in adipocytes. Am J Physiol 273:E46-E51, 1997 (suppl)

7. Shepherd PR, Crowther NJ, Desai M, et al: Altered adipocyte properties in the offspring of protein malnourished rats. Br J Nutr 78:121-129, 1997

8. Hales CN, Desai M, Ozanne SE, et al: Fishing in the stream of diabetes: From measuring insulin to the control of fetal organogenesis. Biochem Soc Trans 24:341-350, 1996

9. Savage PJ, Bennion LJ, Bennett PH: Normalization of insulin and glucagon secretion in ketosis-resistant diabetes mellitus with prolonged diet therapy. J Clin Endocrino Metab 49:830-833, 1979

10. World Health Organization: Diabetes mellitus. Report of a WHO study group. World Health Organ Tech Rep Ser 844:20-25, 1985

11. Yajnik CS, Sardesai BS, Bhat DS, et al: Ketosis resistance in fibrocalculous pancreatic diabetes: II. Hepatic ketogenesis after oral medium chain triglycerides. Metabolism 46:1-4, 1997

12. Keller U, Dchnell H, Sonnenberg GE, et al: Role of glucagon in enhancing ketone body production in ketotic diabetic man. Diabetes 32:387-391, 1983

13. Rao RH: Adaptations in glucose homeostasis during chronic

nutritional deprivation in rats: Hepatic resistance to both insulin and glucagon. Metabolism 44:817-824, 1995

14. Snoeck I, Remacle C, Reusens B, et al: Effect of a low protein diet during pregnancy on the fetal rat endocrine pancreas. Biol Neonate 57:107-118, 1990

15. Stappenbeck R, Hodson AW, Skillen AW, et al: Optimized methods to measure acetoacetate, β-hydroxybutyrate, glycerol, alanine, lactate and glucose using a centrifugal analyzer with a fluorimetric attachment. J Automatic Chem 12:213-220, 1990

16. Proietto J, Nankervis AJ, Traianedes K, et al: Identification of early metabolic defects in diabetes-prone Australian Aborigines. Diabetes Res Clin Pract 17:217-226, 1992

17. Frank JW, Saslow SB, Camilleri M, et al: Mechanism of accelerated gastric emptying of liquids and hyperglycaemia in patients with type II diabetes mellitus. Gastroenterology 109:755-765, 1995

18. Perea A, Clemente F, Martinell J, et al: Physiological effect of glucagon in human isolated adipocytes. Horm Metab Res 27:372-375, 1995

19. McGarry JD: New perspectives in the regulation of ketogenesis. Diabetes 28:517-523, 1979

20. McGarry JD, Foster DW: Regulation of hepatic fatty acid oxidation and ketone body production. Annu Rev Biochem 49:395-420, 1980

21. Boivin A, Deshaies Y: Dietary rat models in which the development of hypertriglyceridemia and that of the insulin resistance are dissociated. Metabolism 44:1540-1547,

22. Arner P, Einarsson K, Ewerth S, et al: Altered action of glucagon on human liver in type 2 (non–insulin-dependent) diabetes mellitus. Diabetologia 30:323-326, 1987