Insulin Resistance During Euglycemic Clamp Studies in Chronically Undernourished Rats With Mild Streptozocin Diabetes

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Malnutrition has been shown to impair insulin sensitivity, but it is not known whether this effect has any impact on coexisting diabetes. Insulin sensitivity was therefore studied using the glucose clamp technique in rats with chronic nutritional deprivation superimposed on mild streptozocin (STZ) diabetes mellitus. In pair-feeding experiments, 4-week-old littermate rats were either allowed ad libitum access to food or restricted to 50% of ad libitum intake for 8 weeks, and were injected with STZ 40 mg/kg intraperitoneally halfway through the experiment. Fasting plasma glucose (FPG) was similar in both groups of rats, but fasting plasma insulin (FPI) was lower in the undernourished group ($P = .016$). Undernourished rats were significantly more insulin resistant during euglycemic hyperinsulinemia of the same degree, with glucose disposal rate being impaired by 50% as compared with that in ad libitum–fed diabetic littermates (24.4 ± 2.8 *v* 51.5 ± 4.4 μmol/kg/min, $P = .0008$). The insulin sensitivity index was significantly lower in the undernourished group (3.03 ± 0.32 *v* 5.67 ± 0.6, $P = .0057$). The results show that chronic undernutrition markedly reduces insulin sensitivity in rats with mild STZ diabetes. This is further evidence that chronic undernutrition is a deleterious modifying influence on coexisting diabetes mellitus. It suggests that the insulin resistance of malnutrition-related diabetes mellitus (MRDM) could potentially be an acquired defect mediated by the coexistent undernutrition, rather than a "distinctive" feature that is intrinsically unique to this diabetic syndrome.

EARLIER STUDIES from this laboratory have shown that chronic balanced nutritional deprivation in rats is characterized by insulin deficiency and insulin resistance.[1,2] These findings are consistent with similar observations in humans.[3-6] Insulin deficiency and insulin resistance also are two characteristic features of malnutrition-related diabetes mellitus (MRDM), comprising unique tropical syndromes regarded as pathogenetically distinct from the two classic forms of diabetes.[7] However, the validity of regarding MRDM as a distinct entity has been questioned.[8] The so-called unique features of MRDM could equally be acquired as a consequence of associated malnutrition, and consequently, patients with MRDM may have nothing more than a nutritionally modified variant of classic diabetes.[9] There is no proof of this at the present time, although there is significant circumstantial evidence that this may be the case.[8,9] Ideally, the proof could be obtained by determining the effect of nutritional rehabilitation on these unique features: their reversibility under such circumstances would be proof of their relationship to coexistent malnutrition. For a variety of socioeconomic reasons, long-term nutritional intervention is extremely difficult in these patients. In the absence of such studies, one way of attempting to resolve the issue is to show experimentally that the metabolic features of malnutrition, such as insulin resistance, are capable of being expressed in the presence of diabetes.

Against this background, the hypothesis was tested that the insulin resistance of malnutrition could modify coexisting diabetes in an animal model of chronic nutritional deprivation induced by balanced food restriction. The details of this model and the importance of differentiating between chronic balanced nutritional deprivation and other forms of malnutrition, such as starvation and severe protein restriction, have been discussed in detail previously.[1,2,10,11] The validity of this pattern (undernutrition) for making comparisons to the commonest forms of human malnutrition has also been discussed, particularly in the context of maintaining stable growth and avoiding growth arrest. In these reports, it has been shown that the insulin deficiency of undernutrition is indeed capable of exacerbating the β-cell dysfunction in diabetes,[10] and that changes in insulin receptor affinity and concentration that are specific to undernutrition[1,12] can be expressed in the presence of coexisting diabetes in this model.[11] In the present study, the significance of these alterations in the context of insulin sensitivity in vivo was studied using the glucose clamp technique to determine whether the insulin resistance of undernutrition could be expressed in the presence of coexisting diabetes. It was thereby hoped to show whether insulin resistance, a so-called unique feature of MRDM, could at least potentially be an acquired defect related to coexistent undernutrition.

MATERIALS AND METHODS

Experimental Animals

Four-week-old littermate male Sprague-Dawley rats (n = 5 in each group) were assigned in pairs to two groups: (1) diabetic controls fed ad libitum for 9 weeks and injected with a subdiabetogenic dose of streptozocin (STZ) 40 mg/kg body weight intraperitoneally in the fifth week (at 8 to 9 weeks of age); and (2) undernourished diabetic animals in whom food was restricted to 50% of ad libitum intake by pair-feeding with diabetic control littermates based on daily weighing of food, and in whom STZ was injected as in the diabetic control group in week 5.

Diet Composition

The diet had a physiological fuel value of 3.3 kcal/g (gross energy value, 4.25 kcal/g), containing 23% protein, 4.5% fat, and 5.8% crude fiber. All vitamins and minerals were provided in excess of the recommended daily allowance (RDA) for growing rats,[13] ensuring that total balanced food restriction did not result in a disproportionate deficiency of any micronutrients. Specifically, dietary concentrations of nutrients known to play a role in glucose

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Submitted August 20, 1994; accepted March 17, 1995.

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0026-0495/95/4411-0010$03.00/0

tolerance were as follows: potassium 1.1% (RDA 0.18%), calcium 1% (RDA 0.5%), zinc 70 ppm (RDA 12 ppm), thiamine 15 ppm (RDA 1.25 ppm), niacin 95 ppm (RDA 15 ppm), and pyridoxine 60 ppm (RDA 7 ppm).

Experimental Protocol

All procedures were authorized by the Institutional Animal Care and Use Committee of the University of Pittsburgh Medical Center. They were maintained on a 12-hour light/dark cycle. Daily food portions were provided for the undernourished rats after 5 PM each evening, to approximate feeding behavior in the two groups (ie, meal-driven in undernourished rats and at onset of darkness in ad libitum–fed animals). On the day before study, food was provided to undernourished animals at 8 AM, based on the overnight intake of the control littermate, and food bins of the latter were emptied as soon as undernourished rats had consumed their portions (usually in an hour). Thus, both groups were fasted for approximately 24 to 26 hours. On the morning of study, rats were anesthetized with pentobarbital 50 mg/kg body weight intraperitoneally, repeated as necessary. The left femoral artery and both femoral veins were cannulated with silastic catheters that were flushed periodically with heparinized saline to maintain patency. Animals were anticoagulated with heparin (25 U/100 g body weight, repeated every 90 minutes). Details of the surgical procedure and sampling protocol have been published previously.[14]

Glucose Clamp Studies

A fasting sample was drawn before commencing the clamp studies, which were performed as previously described in detail.[14] Steady-state plasma glucose of 8 mmol/L was maintained during a constant infusion of insulin at a rate of 1.67 mU/kg/min, using a variable infusion of 25% dextrose that was adjusted based on 5-minute blood samples to maintain plasma glucose at the target value. After steady state was maintained for 30 minutes, three samples were drawn 10 minutes apart. Sampling volumes were based on body weight to equalize the hemodynamic effects of repetitive sampling on animals of widely different body weights.[14] All samples were immediately centrifuged, and plasma was separated into aliquots for subsequent determination of plasma glucose and insulin.

Analytical Methods

Blood glucose level was measured during infusion experiments using an Accu-Chek-II M Blood Glucose Monitor (Boehringer-Mannheim, Indianapolis, IN), to enable rapid adjustments to be made in the glucose infusion rate. Plasma glucose level was measured using a Kodak Ektachem DT60 Glucose Analyzer (Eastman Kodak, Rochester, NY). Plasma insulin was assayed in a double-antibody radioimmunoassay with rat insulin as the standard, as previously described.[1,2,11,14]

Data Analysis

The following calculations were made[15]: glucose disposal rate (μmol/kg/min) = glucose infused in preceding 10 minutes (μmol)/[10 (min) × weight (kg)], and insulin sensitivity index = mean glucose disposal rate over 30 minutes/mean plasma insulin (mean of three values).

Data were analyzed by repeated-measures ANOVA on a VAX 6410 computer (Digital Equipment Corp, Maynard, MA), using the BMDP statistical analysis program.[16] If the omnibus Fisher test for the grouping factor was significant, mean values in the two groups at each time point were then compared with the Neuman-Keuls test. Fasting plasma glucose (FPG) and insulin (FPI) and insulin sensitivity index in undernourished and control animals were compared by *t* tests.

RESULTS

Weight Gain

The rate of weight gain in the two groups is shown in Fig 1. Undernourished diabetic animals grew at a rate that resulted in a body weight that was approximately 50% of that seen in the other groups. Linear regression by groups of mean body weight versus time showed that the slope of the line in ad libitum–fed diabetic rats represented a mean weight gain of 43 ± 1.5 g/wk over the 9 weeks, whereas in undernourished diabetic rats it was 21 ± 0.5 g/wk (multiple $r^2 > .99$ in each case). The difference in slope between the two groups was highly significant statistically ($F_{2,16} = 462$, $P < .00001$). It is particularly noteworthy that undernourished animals did not suffer a period of growth arrest at any point, confirming that a catabolic state was not induced at any stage of the nutritional intervention, in contrast to the pattern seen in starvation or severe protein restriction.

FPG and FPI

FPG was not significantly different in the two groups ($P = .457$). In contrast, FPI was significantly lower in the undernourished group as compared with ad libitum–fed control littermates ($P = .016$). A normal control group of nondiabetic rats was not studied here, but in a previous

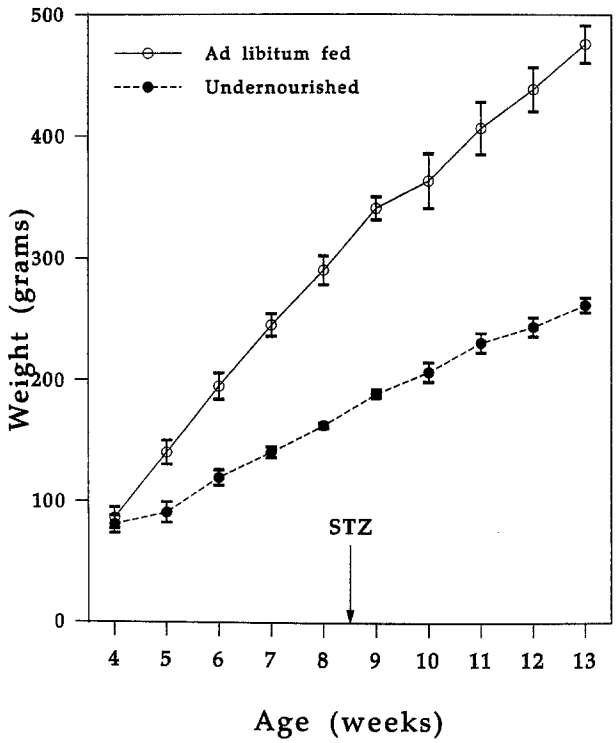

Fig 1. Weight gain over 9 weeks in rats injected with STZ, 40 mg intraperitoneally who were fed ad libitum or were 50% food-restricted to induce chronic undernutrition (n = 5 per group). Growth rates calculated from linear regression were significantly different in the 2 groups ($P < .00005$).

study,[10] it has been shown that ad libitum–fed diabetic rats have fasting levels of glucose that are significantly higher (by ~1.4 mmol/L) and insulin levels that are significantly lower (by 0.15 nmol/L) than those seen in comparably fasted nondiabetic rats. In undernourished diabetic rats, in contrast, plasma glucose is not significantly different from normal, whereas plasma insulin is reduced by 0.6 nmol/L. However, both groups show substantial impairments in glucose tolerance (glucose disappearance rates that are impaired by 40% in ad libitum–fed diabetic rats and 54% in malnourished diabetic rats) (Fig 2).

Glucose Clamp Study

During the sampling period, variation in plasma glucose averaged 7% in both groups (Fig 3). Virtually identical plasma glucose levels were obtained in both groups. Similarly, plasma insulin levels achieved during the clamp studies were not significantly different in the two groups. Repeated-measures ANOVA showed that there was no significant difference between the two groups in insulin values (grouping factor, $P = .255$) and no significant difference at different time points (repeated-measures factor, $P = .2174$) or at individual time points (interaction, $P = .1211$) in the two groups. The fact that the repeated-measures factor was not significant proves that the slight trend toward an increase in undernourished rats and a decrease in ad libitum–fed littermates was not statistically significant, and that steady-state insulin levels were obtained in both groups. Notwithstanding the similar insulin levels, at a steady-state plasma glucose level of 8 mmol/L, undernourished diabetic rats had 50% lower glucose disposal rates ($P = .0008$) as compared with ad libitum–fed diabetic littermates (Fig 3). Insulin sensitivity indices were also significantly different ($P = .0057$), reflecting a marked degree of insulin resistance in the undernourished group (3.1 ± 0.3) versus ad libitum–fed controls (5.7 ± 0.6).

DISCUSSION

The results of this study show that there is a marked impairment of insulin sensitivity under glucose clamp conditions in undernourished diabetic rats as compared with ad libitum–fed rats with STZ diabetes of comparable magnitude. On the surface, this is an apparent contradiction of the observation that fasting glucose was maintained at a lower level by a lower fasting insulin, since this would indicate increased insulin sensitivity. However, static levels of these two variables do not represent the effects of insulin action alone. A variety of counterregulatory hormonal influences other than insulin may also play a role in determining fasting glucose homeostasis, making it hazardous to base any assumptions of insulin action on single time-point estimations of fasting glucose and insulin. Direct evidence for the mechanism underlying this paradox in the malnourished diabetic model is not available, but extrapolation from studies on experimental malnutrition by itself is helpful in shedding some light on this issue, since the identical paradox exists there. In earlier studies, it has been shown that the fasting hypoglycemia of balanced nutritional deprivation is accompanied not only by insulinopenia, but also by insulin resistance, hypoglucagonemia, and glucagon resistance.[4,11,17] Under such circumstances, it is impossible to make any judgments regarding insulin sensitivity without isolating the effects of insulin itself. Even the insulin tolerance test is not ideal for this purpose, since the counterregulatory hormonal response that accompanies the development of hypoglycemia profoundly vitiates the assessment. For these reasons, the glucose clamp technique is generally considered the gold standard for studying the role of insulin action alone,[18] since endogenous insulin production is suppressed by exogenous hyperinsulinemia, and plasma glucose and insulin levels can be equalized to provide a comparable baseline.

Application of the glucose clamp technique to the malnourished diabetic rat model in this study highlights the

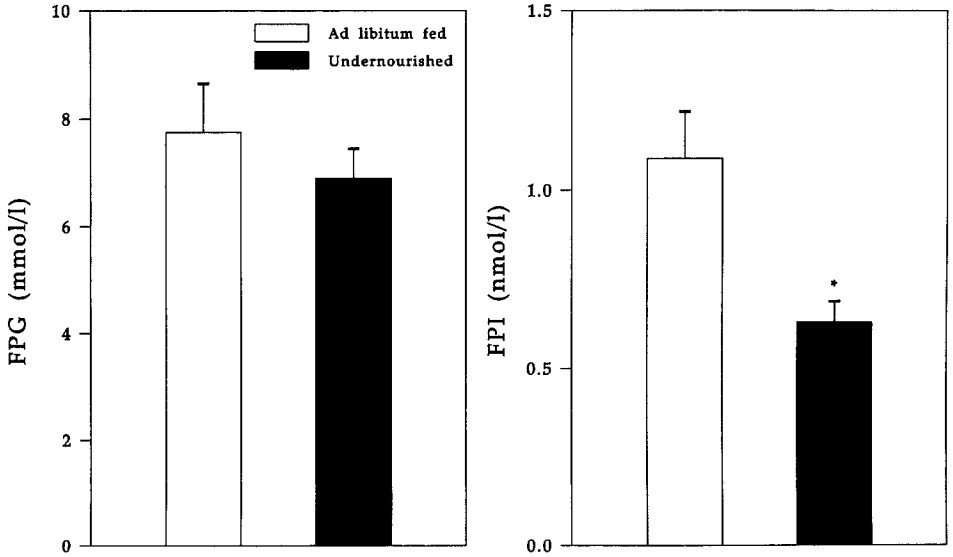

Fig 2. FPG and FPI in diabetic rats (n = 5 per group) fed ad libitum or 50% food-restricted to induce chronic undernutrition. *$P < .02$.

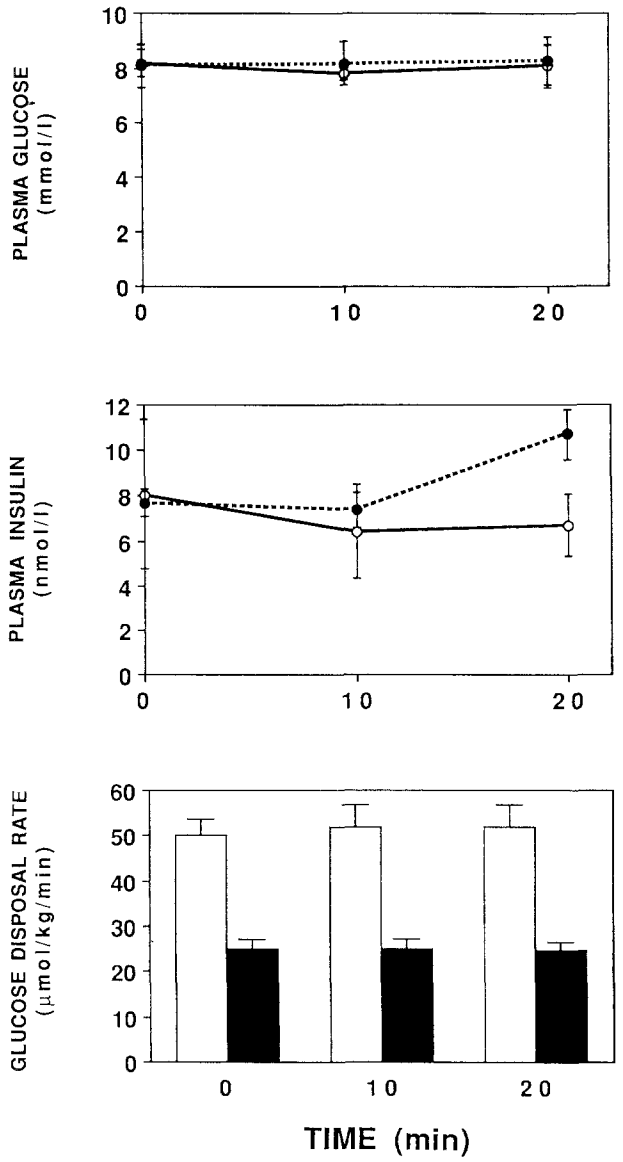

Fig 3. Glucose clamp studies (n = 5 per group). Glucose disposal and plasma insulin while plasma glucose was maintained at steady state (8 mmol/L) with a constant insulin infusion (1.67 mU/kg/min) and a variable glucose infusion in diabetic rats fed ad libitum (○ and □) or 50% food-restricted to induce chronic undernutrition (● and ■). Glucose disposal in the undernourished group was significantly different from control levels in a repeated-measures ANOVA (P = .0008).

importance of not making assumptions based on static levels. It is particularly noteworthy that glucose disposal in undernourished diabetic rats was impaired by 50% during the clamp studies. These findings conclusively establish that the insulin resistance of chronic malnutrition reported previously[2,11] is capable of being expressed in the presence of mild diabetes. They confirm the preliminary observations reported previously using insulin tolerance tests.[11] The insulin resistance cannot be attributed to a more severe degree of hyperglycemia from a greater β-cytotoxic effect of STZ in undernourished rats, because previous studies with glucose tolerance tests have shown that this dose of STZ is not frankly diabetogenic and thus does not produce severe hyperglycemia or severe insulinopenia in either group.[10,11] Moreover, mean levels of glycemia (as reflected in fructosamine levels) have been shown in the past to be similar in the two groups,[10,11] and the effect of malnutrition is almost completely reversible with refeeding, with the STZ-mediated residual deficit being identical to that of ad libitum–fed animals.[10] Thus, insulin resistance of the severity shown here must be attributed to a specific effect of chronic nutritional deprivation, which is itself associated with insulin resistance.[1,2] Consequently, it may be inferred that the insulin resistance of chronic undernutrition can be expressed over and above a background of impaired glucose tolerance in a mildly diabetic animal.

The site at which insulin resistance in malnutrition is mediated cannot be determined from the results of this study. It is possible that it is mediated by a failure of insulin to suppress glucose production in the liver or to stimulate peripheral glucose disposal in skeletal muscle. Glucose turnover studies with radiolabeled glucose are necessary to make the distinction, but it is likely that hepatic glucose production was almost completely suppressed in ad libitum–fed rats at least, based on other studies at a level of hyperinsulinemia similar to that attained here.[19] The insulin resistance seen in undernourished rats could thus be due to incomplete suppression of hepatic glucose production, ineffective stimulation of peripheral glucose disposal, or both.

It is important to distinguish this particular model of undernutrition based on balanced food restriction from malnutrition induced by severe protein restriction. In the latter, in contrast to the results shown here, insulin sensitivity is increased.[20,21] However, the relevance of the protein-restriction model to the vast majority of undernourished individuals in developing countries is doubtful, because severe protein restriction usually results in growth arrest.[22] This is because such individuals continue to grow as children despite the restricted food intake, and as adults, they maintain their weight at a lower than ideal level without developing profound catabolic deficits. The pattern of nutritional deprivation is one of reduced food intake (undernutrition) rather than protein deficiency alone, and the usual pattern of dietary intake is often characterized by a single meal of adequate quality but inadequate quantity to meet the daily requirement.[23,24] In both respects, the balanced nutritional deprivation model closely resembles the human condition. However, it cannot be excluded that additional nutritional deficits commonly present in the human form of malnutrition (eg, vitamin and mineral deficiencies) could also contribute. The purpose of this study was to isolate the effect of caloric deprivation alone, and it is interesting that even when the additional deficits are prevented, malnutrition results in insulin resistance.

In this regard, the insulin resistance of malnutrition appears to be similar to the other abnormality that is associated with it, namely β-cell dysfunction. In this study, as in earlier ones, lower fasting insulin levels were found in undernourished diabetic rats, despite a greater degree of

insulin resistance.[10,11] Using both glucose tolerance tests and hyperglycemic clamp studies, it had been conclusively established that chronic malnutrition amplifies β-cell dysfunction in the mildly diabetic rat. Taken in conjunction with the results of this study, these observations suggest that chronic malnutrition is a deleterious influence on coexisting diabetes, since it accentuates both fundamental defects associated with diabetes: defective insulin secretion and action.

The pathogenetic mechanisms mediating the insulin resistance of undernutrition are not known. One aspect of insulin action at the cellular level known to be affected by chronic nutritional deprivation in this animal model is the binding of insulin to the insulin receptor. It has been shown that insulin binding to erythrocytes and isolated hepatocytes is significantly impaired.[1,12] However, postreceptor events are also probably affected, since the maximal rate of glucose disposal is also impaired in undernourished diabetic rats.[11,25] This phenomenon, known as reduced insulin responsiveness, is said to be indicative of a postreceptor defect in insulin action.[26] The role of metabolites such as ketones and free fatty acids in influencing insulin sensitivity is also not known. In human studies, it has been shown that levels of free fatty acids and ketones are lower in children suffering from protein energy malnutrition than when they have been nutritionally rehabilitated.[27] These metabolites were not measured in this study.

It is unlikely that counterregulatory hormones could have played a major role in mediating the insulin resistance seen in the food-restricted diabetic rats. In previous studies, it has been shown that corticosterone levels are not altered in the model of chronic malnutrition used here.[1] Glucagon levels[17] and growth hormone levels (unpublished data, 1993) are actually reduced in this group of animals. However, it is possible that increased secretion of catecholamines occurs. This aspect of counterregulation has not yet been studied in the rat model of chronic balanced food deprivation used in this study.

Whatever the pathophysiologic basis for the insulin resistance of chronic nutritional deprivation, the demonstration of a defect in insulin action in undernutrition is significant in itself. The results of a recent study suggest that hypoglycemia develops in undernutrition due to impaired gluconeogenesis from glucagon resistance.[2] This may be considered a primary survival adaptation in fuel metabolism that reduces the diversion of protein for use as fuel during nutritional deprivation.[2] In this context, the concomitant development of insulin resistance in a chronically undernourished animal can be thought of as a secondary adaptive response that protects the organism from severe hypoglycemia.[2] It may be hypothesized that in the undernourished diabetic animal, this adaptive process, leading as it does to the development of insulin resistance, plays a futile role that hinders rather than helps the survival of the organism. Although this is speculative, it may be important for the entity called MRDM. MRDM is a diabetic syndrome in which insulin resistance is associated with insulinopenia rather than hyperinsulinemia.[28] The ability to reproduce this combination in an animal model indicates that insulin resistance in that syndrome also may be an acquired defect rather than an intrinsic defect, or a consequence of glucose toxicity.[29] Short-term glycemic control in MRDM does not reduce daily insulin requirements, which often are greater than 2 to 3 U/kg.[28] If the results of the present study can be extrapolated to MRDM, it may be speculated that this is not surprising, since improvements in insulin sensitivity may be dependent on the reversal of malnutrition rather than on the correction of hyperglycemia.

In conclusion, a 50% deficit in food intake substantially impairs the ability of a mildly diabetic rat to respond to insulin. This observation may have important implications in the human context, since it may be applicable to diabetes in undernourished individuals in many parts of the world. It suggests that insulin resistance from undernutrition is capable of being expressed in the presence of coexisting mild diabetes, and that therefore the insulin resistance of MRDM may not necessarily be an integral part of the diabetic syndrome of MRDM, but a consequence of the associated undernutrition. If it is applicable to human diabetes, it would add a new dimension to the "something inherited—something added" hypothesis first proposed by Cerasi and Luft,[30] in which obesity is the only generally recognized nutritional component.

ACKNOWLEDGMENT

The technical assistance of Vince Ballarotto is gratefully acknowledged.

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