Profiling of Zucker Diabetic Fatty Rats in Their Progression to the Overt Diabetic State

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Blood chemistry profiles (glucose, insulin, and triglycerides) and indirect calorimetry were performed on male Zucker diabetic fatty (ZDF) rats in a longitudinal fashion (starting at 7 weeks of age) to assess the nature and timing of specific events in the transition to overt diabetes. Peripheral (skeletal muscle) insulin resistance was clearly present at 7 weeks of age in ZDF rats, yet circulating glucose was only slightly above normal as a result of compensatory hyperinsulinemia. At a crucial stage from 7 to 8 weeks, a reduction in insulin levels instigated several deleterious changes resulting in reduced whole-body carbohydrate utilization and increased glycemia. In subsequent weeks, an inability to sustain peripheral glucose disposal as a consequence of a continuous decline in insulin levels further reduced carbohydrate utilization (increased lipid utilization) and enhanced the overt hyperglycemia. These observations document in a systematic fashion the alterations that define diabetic progression in ZDF rats.

FULL MANIFESTATION of type 2 diabetes mellitus is dependent on the convergence of unidentified genetic abnormalities and deleterious propagating environmental influences. Progress in identifying the genes and factors that drive certain individuals toward diabetes or protect others from developing diabetes is rapidly advancing, in part due to the use of specific animal models of type 2 diabetes. One commonly used animal model of type 2 diabetes is the male Zucker diabetic fatty (ZDF) rat.[1-3] This animal progresses through several stages in the development of diabetes in a relatively predictable age-dependent fashion when maintained under standardized conditions.[1] After weaning (~4 weeks of age), the animals rapidly progress from a hyperinsulinemic-euglycemic (insulin-resistant) state to a hyperglycemic insulin-deficient state (~12 weeks of age).

In the present study, we assessed in a systematic fashion the development of overt diabetes in ZDF rats during their most rapidly changing period (7 to 12 weeks) to gain a better understanding of the nature and timing of specific events. Specifically, the relevant blood chemistry was profiled and the animals were subjected to indirect calorimetry (24-hour measurements) weekly. Through these observations, it was concluded that a subtle decline in the level of hyperinsulinemia between 7 and 8 weeks of age resulted in an inability to sustain glucose entry into the highly insulin-resistant peripheral tissues, causing a reduction in whole-body carbohydrate utilization. These alterations result in severe hyperglycemic progression.

MATERIALS AND METHODS

Animals

All animal procedures were approved by the Institutional Animal Review Board (Eli Lilly and Company, Indianapolis, IN). Male ZDF (n = 6) and Zucker lean (fa/?) (n = 6) rats were obtained from Genetic Models (Indianapolis, IN) at 6 weeks of age. The rats were maintained in an environmentally controlled animal facility (temperature 22°C, 12-hour light/dark cycle) and had free access to food (Purina 5008; PMI Feeds, St Louis, MO) and water.

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Blood Chemistry

On weeks 7, 8, 9, and 12, blood samples were taken from a tail vein of the conscious animals in the fed state. Plasma glucose and triglyceride levels were determined on these samples using a clinical chemistry analyzer (Monarch 2000 Multianalyzer; Instrumentation Laboratories, Lexington, MA). Plasma insulin levels were determined by radioimmunoassay using a rat insulin antibody (Linco Research, St. Charles, MO) and SPA beads (Amersham, Arlington Heights, IL).

Indirect Calorimetry

During a single 24-hour period (starting at 9 AM) on each week of the study, animals were subjected to indirect calorimetry (Oxymax; Columbus Instruments International, Columbus, OH) in a light (12-hour light/dark cycle, lights on at 6 AM) and temperature (24°C)-controlled environment. The animals were maintained with free access to food and water throughout the duration of the 24-hour measurement period. Oxygen consumption ($\dot{V}O_2$) was calculated as the ventilation rate (L/min) · fraction of O_2 in the chamber-ventilation rate (L/min) · fraction of O_2 leaving the chamber. CO_2 production ($\dot{V}CO_2$) was calculated as the ventilation rate (L/min) · fraction of CO_2 leaving the chamber-ventilation rate (L/min) · fraction of CO_2 in the chamber. The respiratory quotient (RQ) is the ratio of $\dot{V}CO_2$ to $\dot{V}O_2$.

Isolated Muscle Preparation

Isolated muscle glucose transport was assessed in a parallel group of ZDF and lean Zucker rats. At 7 weeks of age, ZDF and lean Zucker rats were anesthetized with pentobarbital sodium (6 mg/100 g body weight) in the fed state. Fast-twitch epitrochlearis muscle and slow-twitch soleus muscle strips were then isolated and incubated for assessment of basal and submaximal (600 pmol/L) insulin-stimulated glucose transport as described previously.[4]

Statistical Analysis

The results are expressed as the mean ± SE. Data were analyzed by ANOVA, with significant differences between means identified using Fisher's protected least-significant difference. Differences were considered significant at a P level less than .05.

RESULTS

Animals

ZDF rats averaged 284.78 ± 6.55 g body weight at the beginning of the study (7 weeks of age), whereas lean rats were

192.50 ± 4.22 g. At the termination of the study (12 weeks of age), ZDF rats averaged 397.70 ± 6.60 g body weight, whereas lean rats were 302.31 ± 5.13 g.

Plasma Glucose

Plasma glucose levels at 4 different ages are displayed in Fig 1 for both lean and ZDF rats. At 7 weeks of age, plasma glucose levels were slightly higher in ZDF rats versus lean control rats (9.38 ± 0.65 *v* 7.38 ± 0.18 mmol/L, $P = .014$). At the later time points, plasma glucose increased significantly and steadily in ZDF rats, reaching a maximal value of approximately 31.5 mmol/L at the 12-week time point. Plasma glucose levels did not change from week 7 to week 9 in lean rats.

Plasma Triglycerides

Plasma triglyceride levels at 7 to 9 weeks of age are displayed in Fig 2 for both lean and ZDF rats. At 7 weeks of age, plasma triglycerides were significantly higher in ZDF rats versus lean controls (5.59 ± 0.45 *v* 1.27 ± 0.12 mmol/L, $P < .0001$). Plasma triglycerides increased significantly in ZDF rats from 7 to 8 weeks (5.59 ± 0.45 *v* 9.48 ± 0.97 mmol/L, $P < .0001$), but did not increase further thereafter. Plasma triglycerides did not change from weeks 7 to 9 in lean rats.

Plasma Insulin

Plasma insulin levels at 4 different ages are displayed in Fig 3 for both lean and ZDF rats. Plasma insulin levels did not change significantly from weeks 7 to 9 in lean rats, and thus for comparisons with ZDF rats at different ages, the mean value for lean rats was used (0.275 ± 0.12 nmol/L). At 7 weeks, plasma insulin in ZDF rats (5.49 ± 0.54 nmol/L) was approximately 20-fold the mean lean value ($P < .0001$). ZDF insulin levels decreased from weeks 7 to 8 (5.49 ± 0.54 *v* 3.52 ± 0.49 nmol/L, $P = .001$) but remained at approximately 13-fold the mean lean value ($P < .0001$). From 8 to 9 weeks, the reduction in ZDF insulin levels was not statistically significant ($P = .09$).

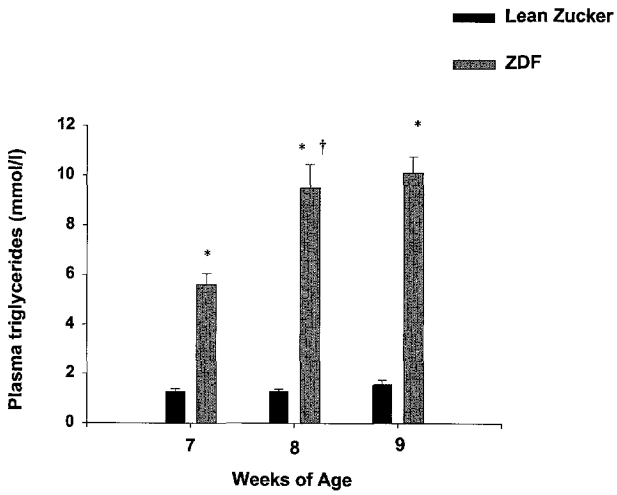

Fig 2. Plasma triglyceride levels in lean Zucker (n = 6) and ZDF (n = 6) rats at the indicated ages. *Significantly different *v* lean, †significantly different *v* preceding week ZDF.

At 12 weeks, ZDF insulin levels declined further but remained well above the lean levels (1.36 ± 0.21 *v* 0.275 ± 0.12 nmol/L, $P < .0001$).

RQ Measurements

Age 7 weeks. RQ measurements from both lean and ZDF rats for week 7 are displayed in Fig 4A. The RQ tended to be near unity in ZDF rats throughout the duration of the 24-hour period, whereas in lean rats, values were between 0.85 and 0.95 (mean, 0.91 ± 0.007). An analysis of the compiled data showed a significant difference between the mean RQs for ZDF versus lean rats over the 24-hour period (0.98 ± 0.004 *v* 0.91 ± 0.007, $P < .0001$).

Age 8 weeks. RQ measurements for both lean and ZDF rats for week 8 are displayed in Fig 4B. In lean rats, the mean RQ did not differ at week 8 versus week 7 (0.92 ± 0.004 *v* 0.91 ± 0.007, $P = .57$). In contrast, the mean ZDF RQ declined significantly from 0.98 ± 0.004 to 0.94 ± 0.01 ($P = .001$).

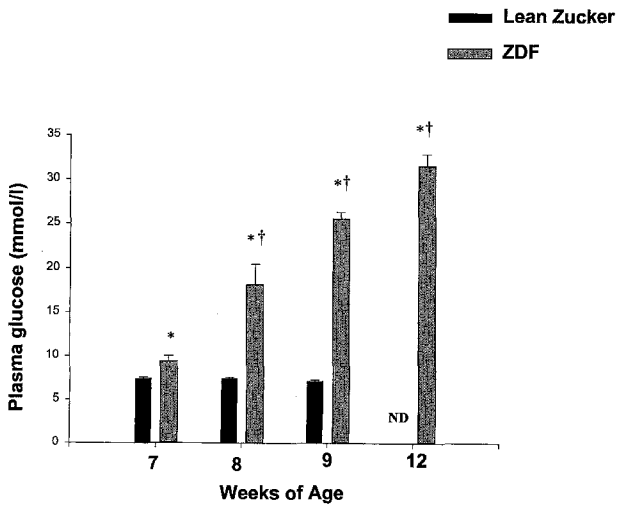

Fig 1. Plasma glucose levels in lean Zucker (n = 6) and ZDF (n = 6) rats at the indicated ages. Plasma glucose was not determined (ND) in 12-week-old lean rats. *Significantly different *v* lean, †significantly different *v* preceding week ZDF.

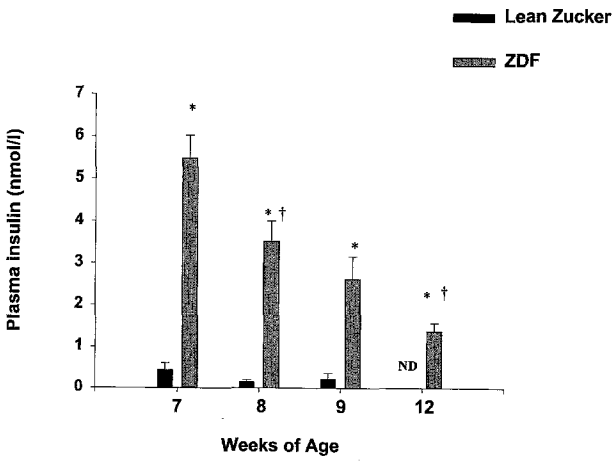

Fig 3. Plasma insulin levels in lean Zucker (n = 6) and ZDF (n = 6) rats at the indicated ages. Plasma insulin was not determined (ND) in 12-week-old lean rats. *Significantly different *v* lean, †significantly different *v* preceding week ZDF.

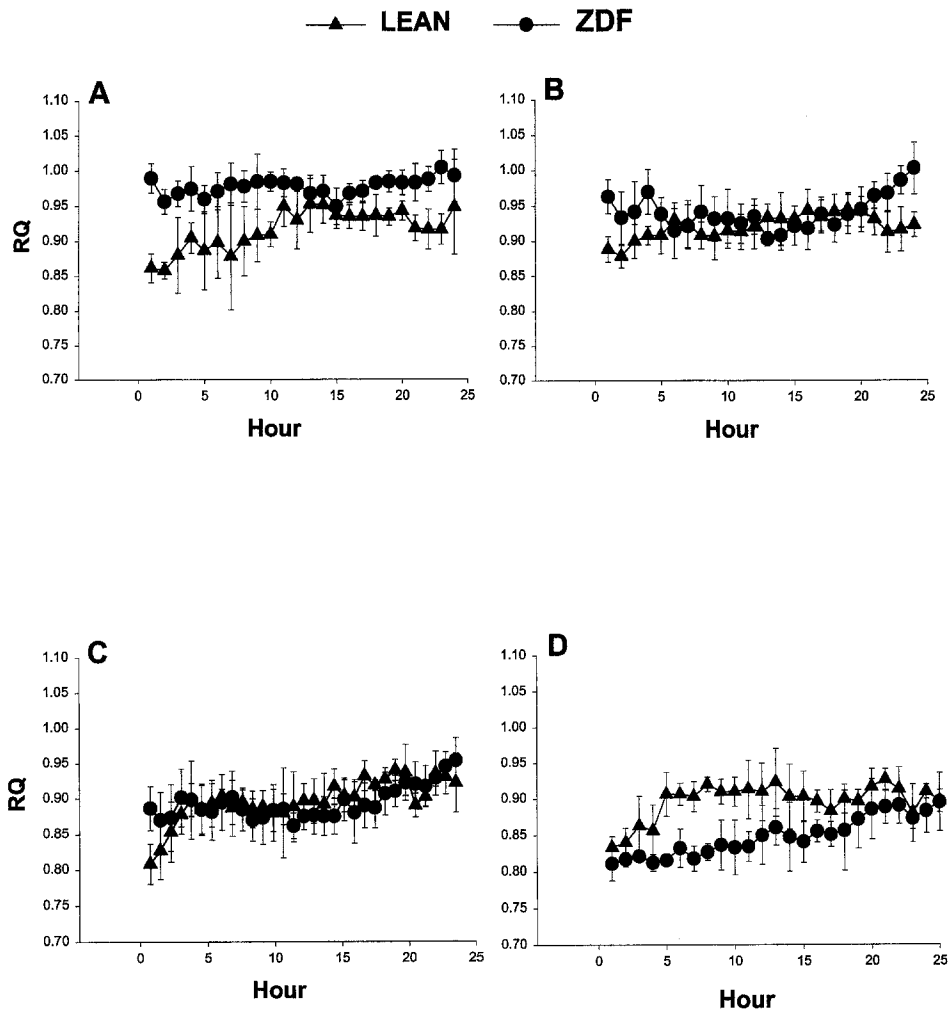

Fig 4. RQ assessed hourly over a 24-hour period at age 7 weeks (A), 8 weeks (B), 9 weeks (C), and 12 weeks (D) for both lean Zucker (n = 6) and ZDF (n = 6) rats.

Furthermore, at week 8, the mean RQ was no longer significantly different between ZDF and lean rats (0.94 ± 0.01 *v* 0.92 ± 0.004, $P = .066$).

Age 9 weeks. RQ measurements for both lean and ZDF rats for week 9 are displayed in Fig 4C. The RQ in lean rats did not differ significantly from the initial measurements ($P = .17$ *v* week 7). However, the mean ZDF RQ continued to decline, showing a significant reduction from the week 8 measurement (0.89 ± 0.01 *v* 0.94 ± 0.01, $P < .0001$). The week 9 RQ for lean and ZDF rats did not differ significantly ($P = .50$).

Age 12 weeks. RQ measurements for both lean and ZDF rats for week 12 are displayed in Fig 4D. The RQ in lean rats did not differ significantly from the initial measurements ($P = .08$ *v* week 7). The mean ZDF RQ reached the lowest level at week 12 and was significantly different versus all previous measurements for either lean or ZDF rats ($P < .0001$ for all comparisons).

Regression Analyses

A series of regression analyses on the data from ZDF rats were performed to investigate potential relationships among the variables (RQ, insulin, glucose, and triglycerides). The RQ was significantly correlated with insulin levels in ZDF rats ($r = .76$; $P < .0001$; Fig 5A). The RQ also demonstrated a significant inverse relationship with glucose ($r = .90$, $P < .0001$; Fig 5B) and triglycerides ($r = .67$, $P = .003$; Fig 5C). Glucose levels in ZDF rats were inversely related to insulin levels ($r = .69$, $P = .001$; Fig 5D) and positively related to triglyceride levels ($r = .61$, $P = .007$; Fig 5E). Finally, insulin levels demonstrated a weak inverse relationship with triglyceride levels ($r = .54$, $P = .02$; Fig 5F).

Skeletal Muscle Glucose Transport

3-*O*-Methyl-D-glucose transport measurements in fast-twitch epitrochlearis and slow-twitch soleus muscle strips from 7-week-old ZDF and lean Zucker rats are presented in Fig 6A and B. In epitrochlearis from lean rats, a submaximal insulin stimulus (600 pmol/L) approximately doubled the glucose transport activity. In contrast, glucose transport was essentially unchanged in ZDF epitrochlearis exposed to 600 pmol/L insulin. A very similar pattern was observed in soleus muscle strips, where submaximal insulin increased the transport activity approximately 2.2-fold in lean rats but had essentially no effect in ZDF rats.

DISCUSSION

ZDF rats progress rapidly from an insulin-resistant prediabetic state to a diabetic condition characterized by hyperglycemia and disproportionately low insulin levels.[1] In contrast, the

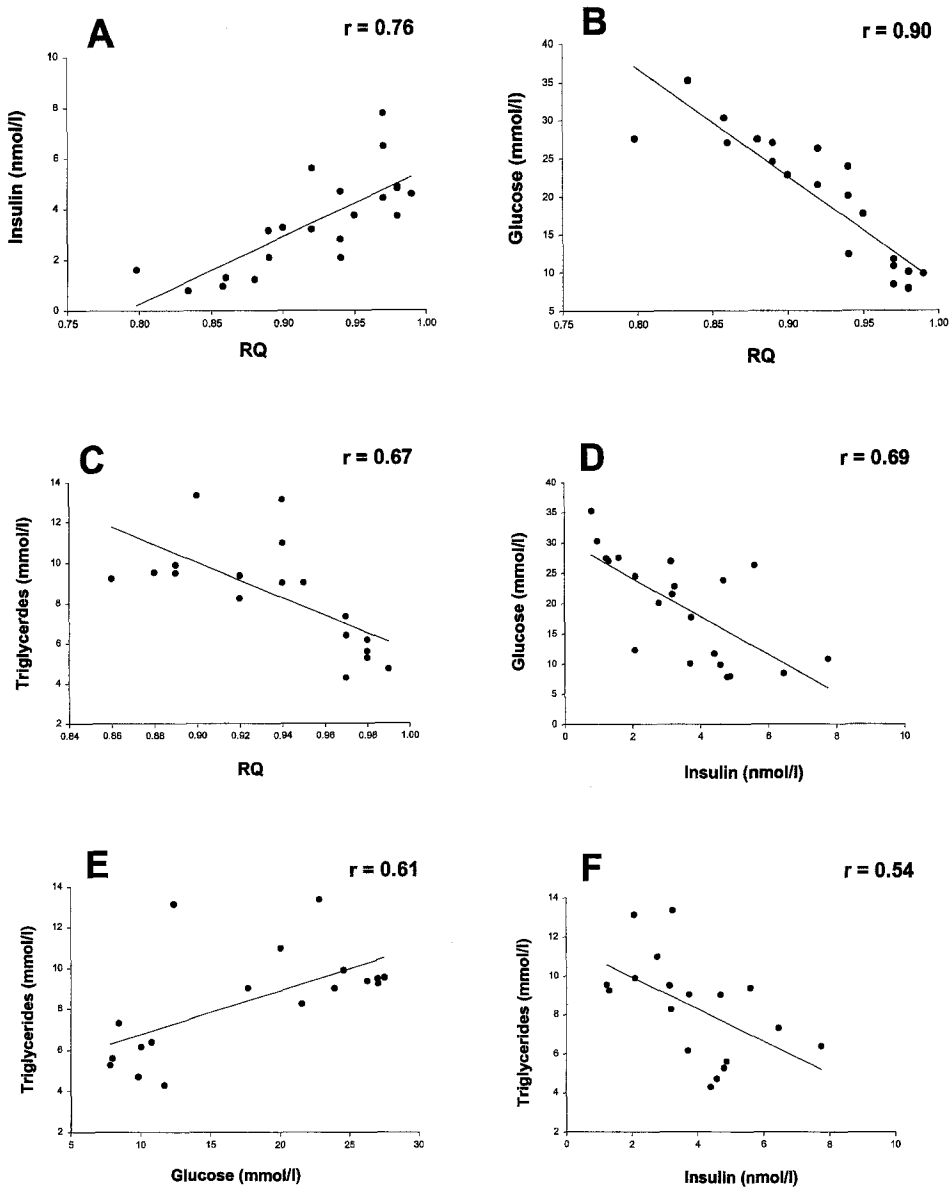

Fig 5. Regression analyses on select variables from ZDF rats for examination of relationships between insulin and RQ (A), glucose and RQ (B), triglycerides and RQ (C), glucose and insulin (D), triglycerides and glucose (E), and triglycerides and insulin (F).

nondiabetic obese fa/fa Zucker rat avoids the development of hyperglycemia and frank diabetes despite peripheral insulin resistance similar to the ZDF rat,[4] most probably because of sustained compensatory hyperinsulinemia. In the present study, we evaluated changes in the blood chemistry profile and RQ during an especially volatile period in the ZDF progression toward overt diabetes. Prior to 8 weeks of age, ZDF rats clearly display peripheral (skeletal muscle) insulin resistance but maintain near-normal glucose levels through a compensatory elevation in circulating insulin levels. It may also be suggested that hyperglycemia is prevented at this early stage by an enhanced utilization of carbohydrate as RQ levels approached unity, indicating nearly exclusive use of carbohydrate as the fuel source. The choice of carbohydrate as the primary fuel source is predictably driven by the insulin levels, and it is this feature that provides valuable insight into the spiraling hyperglycemia that occurs over a few weeks' time.

Over the course of the experimental measurements, the period from week 7 to week 8 appears most critical. During this time, several important changes occur that initiate the manifestation of overt diabetes, the most relevant being a slight yet highly significant reduction in insulin levels. From weeks 7 to 8, insulin levels decreased from approximately 20-fold above normal to 13-fold above normal. Thus, circulating insulin levels were still extremely high relative to the controls, yet apparently were not high enough to maintain peripheral glucose uptake and utilization as evident by the significant reduction in the RQ noted from week 7 to week 8. Regression analyses performed among insulin, glucose, and the RQ from ZDF rats illustrate the importance of the relationships between these variables in the development of diabetes.

As already mentioned, the hyperinsulinemia in ZDF rats at 7 weeks was accompanied by very high rates of carbohydrate utilization (RQ). As insulin levels declined in the following weeks, carbohydrate utilization declined steadily, and thus a significant positive correlation between insulin and the RQ was

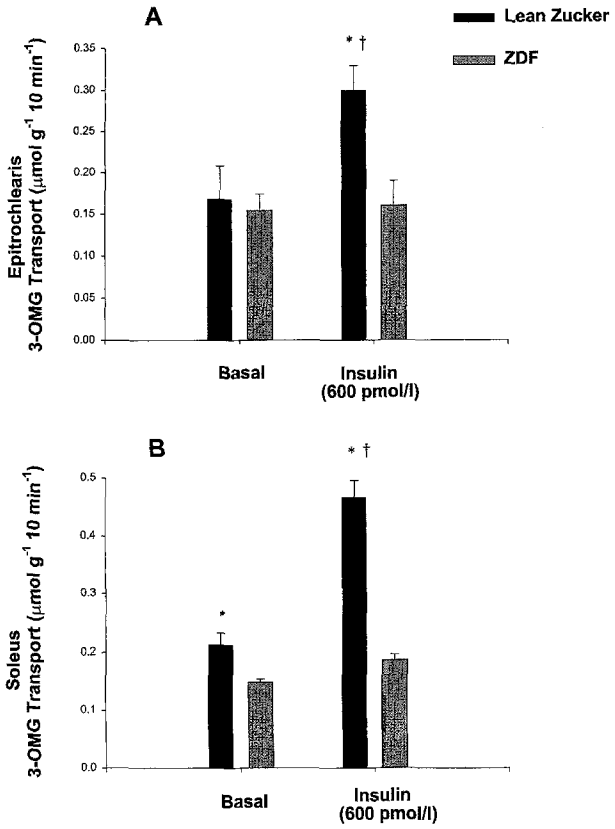

Fig 6. 3-*O*-Methyl-D-glucose (3-OMG) transport measurements in fast-twitch epitrochlearis (A) and slow-twitch soleus (B) from both lean Zucker and ZDF rats. 3-OMG transport was assessed in the absence of insulin and in the presence of a submaximal insulin stimulus (600 pmol/L, n = 6 for each condition). *Significantly different *v* correspondingly treated ZDF, †significantly different *v* lean basal.

observed in ZDF rats. As the insulin level and RQ declined, glucose levels increased dramatically. The elevation in glucose was most certainly the consequence of insufficient insulin to drive glucose disposal in the insulin-resistant peripheral tissue and an inability to suppress hepatic glucose output, which explains the significant inverse correlation between insulin and glucose. Furthermore, it is clear that if glucose cannot be transported into peripheral tissues, the utilization of carbohydrate, as reflected in the RQ, declines. Thus, the highly significant inverse correlation observed between the RQ and glucose can be explained, at least in part, by the declining ability to move glucose into tissues for disposal. As the age of the ZDF rats increased, further reductions in insulin levels were observed, and thus carbohydrate utilization (RQ) continued to decline while plasma glucose increased.

These observations point toward a conglomeration of factors that must be present for the progression of diabetes in ZDF rats. Peripheral insulin resistance necessitates hyperinsulinemia in order to maintain glucose homeostasis. However, if the β-cells falter and cannot provide the requisite insulin, glucose utilization by peripheral tissues declines, thereby escalating circulating glucose. These events exacerbate the problem, as increased fat utilization (decreased RQ) and increased circulating glucose both further reduce glucose-induced insulin release and peripheral insulin sensitivity,[5-7] making it more difficult to remove glucose from the circulation. Thus, it is evident that the β-cell failure in the face of insulin resistance initiates and sustains the development of hyperglycemia in ZDF rats. The evolution of pancreatic β-cell failure in ZDF rats has been previously attributed to excessive pancreatic triglyceride accumulation.[8-10] In the current study, plasma triglyceride levels were significantly elevated at all time points examined and demonstrate a significant inverse relationship with plasma insulin, in agreement with the "lipid toxicity" hypothesis.

In summary, before 8 weeks of age, hyperinsulinemic ZDF rats do not demonstrate a hyperglycemic profile despite severe skeletal muscle insulin resistance. However, at 8 weeks of age, a reduction in insulin levels, possibly resulting from excess islet lipid accumulation, disrupts the delicate balance between insulin and peripheral glucose utilization. This reduction in insulin, albeit to levels substantially greater than those in control lean rats, results in an inability to adequately suppress hepatic glucose production and maintain high-level glucose uptake and disposal, thus producing hyperglycemic progression.

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