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β-Cell lipotoxicity in the pathogenesis of non-insulin-dependent diabetes mellitus of obese rats: Impairment in adipocyte–β-cell relationships

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ABSTRACT **Hyperinsulinemia, loss of glucose-stimulated insulin secretion (GSIS), and peripheral insulin resistance coexist in non-insulin-dependent diabetes mellitus (NIDDM). Because free fatty acids (FFA) can induce these same abnormalities, we studied their role in the pathogenesis of the NIDDM of obese Zucker diabetic fatty (ZDF-drt) rats from 5 weeks of age (before the onset of hyperglycemia) until 14 weeks. Two weeks prior to hyperglycemia, plasma FFA began to rise progressively, averaging 1.9 ± 0.06 mM at the onset of hyperglycemia ($P < 0.001$ vs. controls). At this time GSIS was absent and β-cell GLUT-2 glucose transporter was decreased. The triacylglycerol content of prediabetic islets rose to 10 times that of controls and was correlated with plasma FFA ($r = 0.825$; $P < 0.001$), which, in turn, was correlated with the plasma glucose concentration ($r = 0.873$; $P < 0.001$). Reduction of hyperlipacidemia to 1.3 ± 0.07 mM by pair feeding with lean littermates reduced all β-cell abnormalities and prevented hyperglycemia. Normal rat islets that had been cultured for 7 days in medium containing 2 mM FFA exhibited increased basal insulin secretion at 3 mM glucose, and first-phase GSIS was reduced by 68%; in prediabetic islets, first-phase GSIS was reduced by 69% by FFA. The results suggest a role for hyperlipacidemia in the pathogenesis of NIDDM; resistance to insulin-mediated antilipolysis is invoked to explain the high FFA despite hyperinsulinemia, and sensitivity of β cells to hyperlipacedemia is invoked to explain the FFA-induced loss of GSIS.**

Despite decades of intensive research the pathogenesis of non-insulin-dependent diabetes mellitus (NIDDM), a disorder that affects 2–5% of the world's population, remains obscure. Because it may precede the onset of hyperglycemia by many years, insulin resistance is widely viewed as the primary abnormality in the disease (1). In this formulation the associated hyperinsulinemia is viewed as a secondary compensation by β cells for the antecedent insulin insensitivity; when hyperglycemia begins, it is regarded as reflecting an inability of hypersecreting β cells to meet an ever-increasing insulin requirement (2). However, neither the mechanism by which β cells initially maintain a high enough level of insulin secretion to prevent hyperglycemia despite increasing insulin resistance nor the cause of their ultimate failure to do so has been identified. β-Cell failure is accompanied in human and rodent NIDDM by complete loss of glucose-stimulated insulin secretion (GSIS) (3, 4) and, in all rodent models thus far studied, by a parallel reduction in β cells displaying GLUT-2, the high-K_m facilitative glucose transporter (4–7).

Long-chain fatty acids, which may be central to the development of insulin resistance in NIDDM (8, 9), can stimulate basal insulin secretion (10–13) and inhibit GSIS in isolated islets (13–17). This suggests a scheme that could account for the β-cell abnormalities in pre-NIDDM and NIDDM and explain the relationship between insulin resistance and β-cell dysfunction. In this model the increase in plasma free fatty acids (FFAs) associated with prediabetic obesity causes both the insulin resistance and the matching hyperinsulinemia of the prediabetic state; subsequently a further increase in FFAs causes the β-cell unresponsiveness to hyperglycemia that characterizes overt diabetes.

The present study tests this hypothesis in a rodent model of obesity-associated NIDDM that most closely resembles the human disorder, the Zucker diabetic fatty (ZDF-drt) rat. NIDDM begins in almost 100% of obese male ZDF rats (*fa/fa*) between 7 and 10 weeks of age, while virtually all obese female ZDF rats (*fa/fa*) remain nondiabetic (17). This permits early identification of prediabetic and nonprediabetic littermates. It is, therefore, possible to test the propositions that the hyperinsulinemia of the compensated prediabetic phase results, at least in part, from a moderate rise in plasma FFA levels and that the later loss of GSIS is caused by a further increase in FFAs to a critical concentration that blocks the β-cell response to glucose.

MATERIALS AND METHODS

Animals. Four groups of rats were studied: obese male ZDF prediabetic and diabetic rats (*fa/fa*), obese female ZDF nondiabetic rats (*fa/fa*), nonobese male ZDF littermates (*fa/+* or *+/+*), and male Wistar rats. Normal Wistar rats were obtained from Sasco (Omaha). Homozygous obese ZDF-drt rats (*fa/fa*) and lean ZDF littermates (*fa/+* or *+/+*) were bred in our laboratory from [ZDF/Drt-*fa* (F10)] rats purchased from R. Peterson (University of Indiana School of Medicine, Indianapolis). Our colony exhibits the same phenotype as was previously described (17). Obesity is discernible at ≈4 weeks of age, and all obese homozygous male rats developed hyperglycemia (blood glucose > 200 mg/dl), glycosuria, hyperinsulinemia, and hyperlipidemia by 8–10 weeks of age. Consequently, at 6 weeks all obese males could be considered to be prediabetic. By contrast, obese homozygous females developed hyperinsulinemia and hyperlipidemia but not hyperglycemia. All rats received standard rat chow (Teklad F6 8664, Teklad, Madison, WI) ad libitum and had free access to water. A subset of obese prediabetic male rats were pair fed with lean littermates.

Plasma Measurements. Blood samples were collected at approximately 9:00 a.m. from tail veins into capillary tubes coated with EDTA. Plasma glucose was measured by the

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Abbreviations: BSA, bovine serum albumin; FFA, free fatty acid; GSIS, glucose-stimulated insulin secretion; IRI, immunoreactive insulin; NIDDM, non-insulin-dependent diabetes mellitus; TG, triacylglycerol.

glucose oxidase method using the glucose analyzer II (Beckman). Plasma FFAs were determined with kits (Boehringer Mannheim). Plasma triacylglycerols (TGs) were measured with a Sigma diagnostic kit (GPO-Trinder procedure no. 337); TGs were hydrolyzed by lipase to glycerol and FFAs, and the glycerol was assayed by coupled enzyme reactions catalyzed by glycerol kinase, glycerol phosphate oxidase, and peroxidase (18). Immunoreactive insulin (IRI) was determined by radioimmunoassay (19) using charcoal separation (20).

Pancreatic Perfusion. Pancreata were isolated and perfused for 40 min by the method of Grodsky and Fanska (21) as modified (22). Our standard perfusate, Krebs–Ringer bicarbonate buffer (pH 7.6) containing 5.6 mM glucose (to simulate the normal fasting glucose level) and 5 mM pyruvate, 5 mM fumarate, and 5 mM glutamate, was perfused throughout all experiments. The added substrates do not by themselves alter basal or stimulated insulin secretion. After a 10-min baseline period during which the standard solution was perfused, 14.4 mM D-glucose was coperfused via a side-arm catheter for 10 min, raising the total glucose concentration in the perfusate to 20 mM. Samples were collected at 1-min intervals and stored at −20°C until assay. Five or six perfusion experiments were carried out in each of the four rat groups at 6 weeks and 12 weeks of age. The IRI increment during stimulation was calculated by subtracting the true baseline (mean of the IRI values during the initial 10 min before stimulation) from the mean of the IRI values (microunits/ml per min) during each stimulatory period.

Morphometry. Bouin-fixed paraffin-embedded serial sections of perfused pancreata (5-μm thickness) were stained for insulin and GLUT-2 by indirect immunofluorescence (23). The percentage of GLUT-2-positive β cells was determined from the ratio of the area of GLUT-2-positive to that of insulin-positive cells (24).

TG Content of Islets. Isolated islets were counted under the microscope. Fifty microliters of 2 mM NaCl/20 mM EDTA/50 mM sodium phosphate buffer, pH 7.4, was added to 100–300 islets, which were then sonicated for 1–2 min. Then 10 μl of homogenate was mixed with 10 μl of *tert*-butyl alcohol and 5 μl of Triton X-100/methyl alcohol mixture (1:1 by volume) for the extraction of lipids. TGs were measured with a Sigma diagnostic kit.

Perifusion of Cultured Islets. Islets were isolated by a modification (25) of the method of Naber *et al.* (26) and were maintained in suspension culture in 60-mm glass Petri dishes at 37°C in a humidified atmosphere of 5% CO_2/95% air. The culture medium consisted of RPMI 1640 supplemented with 10% fetal bovine serum, penicillin (200 units/ml), streptomycin (0.2 mg/ml; GIBCO/BRL), and 2% bovine serum albumin (BSA, fraction V; Miles), either with or without 2 mM long-chain fatty acids (oleate/palmitate, 2:1, sodium salt; Sigma). The medium was changed on the day after isolation and every second day thereafter. The final glucose concentration of the medium was 9.4 mM. This concentration is required to maintain optimal long-term survival of islets (>90%).

For perifusion, 50–100 islets were picked up under a stereoscopic microscope, washed with phosphate-buffered saline, equilibrated in a Hepes/bicarbonate-buffered salt solution containing 3 mM glucose for 20 min, and loaded into 13-mm chambers containing 8-μm nylon membranes (Millipore). The closed chambers were suspended in a water bath set at 37°C. Islets were perifused with buffer containing 3 mM glucose. Flow rate was maintained at 0.8 ml/min with a peristaltic pump, and effluent fractions were collected at 2-min intervals into chilled tubes containing benzamidine (30 mM; Aldrich) and stored at −20°C until insulin assay.

RESULTS

Plasma FFA Levels in Prediabetic Rats During the Development of NIDDM. The validity of the hypothesis outlined in the Introduction requires that significant hyperlipacidemia be present in prediabetic rats prior to the appearance of hyperglycemia. We therefore obtained weekly measurements of free FFAs and TGs in the plasma of obese male ZDF prediabetic rats, using female homozygotes and male heterozygotes as obese and nonobese controls. Sampling began at 5 weeks of age—i.e., 3–4 weeks before the onset of overt diabetes—and continued until 14 weeks of age, at which time overt diabetes had been present for at least 4 weeks (Fig. 1). Compared with lean controls, plasma TG levels were slightly higher after the age of 7 weeks in both prediabetic and nondiabetic obese groups (Fig. 1*B*). But the most striking finding was in the prediabetic males; at 7 weeks of age, ≈2 weeks before the appearance of hyperglycemia, their mean FFA level had risen to 1.2 ± 0.05 mM, significantly greater than in the obese and lean controls ($P < 0.001$), and was 1.9 ± 0.06 mM at 10 weeks of age, several days after the onset of hyperglycemia (>11.0 mM glucose) (Fig. 1*C*). Thus, islets of prediabetic rats were exposed to higher plasma FFA levels than their obese and lean littermates for an average of 2 weeks before the onset of hyperglycemia. There was a significant correlation between the morning blood glucose levels and the plasma levels of FFAs of Fig. 1 ($r = 0.873$; $P < 0.001$); at the onset of overt NIDDM plasma FFAs exceeded 1.5 mM in every rat.

Islet TG Content in Diabetes. We reasoned that if fatty acyl CoA in islets increases in proportion to the plasma levels of

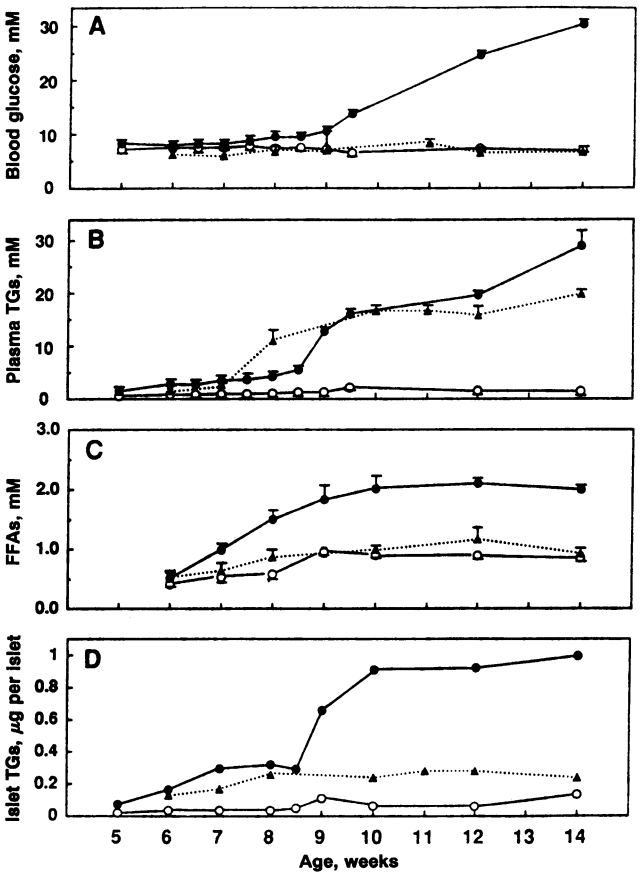

FIG. 1. Longitudinal studies of plasma glucose (*A*), plasma TG (*B*) and FFA (*C*) levels, and TG content of islets (*D*) in lean male ZDF rats (*fa/+*) (○); obese female ZDF rats (*fa/fa*) (▲), which do not develop diabetes; and obese male ZDF rats (*fa/fa*) (●), which develop diabetes between the ages of 8 and 10 weeks.

Table 1. Effects of 6 weeks of pair feeding of prediabetic ZDF rats with lean littermates, beginning at 6 weeks of age

	Body weight, g	Plasma			Islets			
		FFAs, mM	TGs, mM	Glucose, mM	TGs, μg per islet	Basal IRI, μU/ml	Glucose stimulated IRI, μU/ml	GLUT-2 positivity, %
ZDF (*fa/fa*) ♂, ad libitum	492 ± 10	2.10 ± 0.02	22.60 ± 0.23	24.60 ± 0.44	0.99 ± 0.03	225.7 ± 22.4	0	25.1 ± 6.4
ZDF (*fa/fa*) ♂, pair fed	351 ± 17*	1.30 ± 0.07*	3.45 ± 0.43*	6.60 ± 0.17*	0.24 ± 0.05*	71.6 ± 12.9	61.0 ± 3.9	90.3 ± 3.6
ZDF (*fa/+*) ♂, ad libitum	361 ± 4	0.89 ± 0.04	2.43 ± 0.04	5.70 ± 0.17	0.06 ± 0.01	7.7 ± 1.8	93.5 ± 11.9	98.5 ± 2.5
ZDF (*fa/fa*) ♀, ad libitum	415 ± 8	1.09 ± 0.10	18.20 ± 2.02	9.15 ± 0.02	0.24 ± 0.01	141.2 ± 7.1	117.9 ± 18.8	98.5 ± 1.6

The IRI values represent the mean of four to six perfusion experiments for each group. Basal IRI or glucose-stimulated IRI represents the average of 10 insulin measurements in the effluent of the pancreas collected at 1-min intervals for 10 min during perfusion with a glucose concentration of 5.6 mM (basal) or 20 mM (glucose-stimulated). μU, microunits.
*$P < 0.01$ vs. ZDF (*fa/fa*) ♂, ad libitum. Statistical analyses were performed by Student's *t* test for two groups.

FFAs, in the presence of sufficient glycerol 3-phosphate for reesterification of the fatty acids, secondary TG formation might take place in the islets. A reduction in β-cell glycerol-3-phosphate shuttle activity (27), which has been reported in two other animal models of NIDDM (28, 29), would increase glycerol 3-phosphate, a requirement for TG synthesis. We therefore measured islet TG content. In islets of prediabetic ZDF rats, TGs had increased 4-fold between the ages of 5 and 8.5 weeks (Fig. 1*D*). An additional, abrupt 2-fold increase occurred by 9 weeks of age, when the mean glucose level had reached 10.6 mM (Fig. 1*D*). At 10 weeks islet TG content reached a plateau that was 10 times higher than in 5-week-old prediabetic ZDF rats and 10-week-old lean littermates and 4 times higher than in 10-week-old obese nondiabetic female littermates. There was a significant correlation between plasma FFA and islet TG levels ($r = 0.825$; $P < 0.001$), consistent with reesterification of fatty acids in islets. Islet TG content was also correlated with blood glucose levels ($r = 0.929$; $P < 0.001$).

Effects of Reduction of Hyperlipacidemia on β-Cell Phenotype of Prediabetic Rats. To determine whether a decrease in the elevated plasma FFA levels would prevent the β-cell abnormalities in this form of NIDDM, we restricted the caloric intake of prediabetic rats, a maneuver that substantially reduces the hyperlipacidemia of obese animals. Prediabetic ZDF rats were pair fed with lean littermates beginning at 6 weeks of age and continuing until the age of 12 weeks. As shown in Table 1, the moderate reduction in plasma FFAs was associated with marked attenuation of the entire phenotype of NIDDM—i.e., the hyperglycemia, hypertriacylglycerolemia, accumulation of fat in islets, and loss of β-cell GLUT-2 glucose transporter. Basal hyperinsulinemia was reduced by 60%, to below the levels of obese nondiabetic female ZDF controls, and GSIS was preserved at about half of the normal level in nondiabetic controls. The loss of GLUT-2 was prevented.

Effects of Long-Chain Fatty Acids on Cultured Islets. The foregoing results are consistent with a role for long-chain fatty acids in the pathogenesis of the NIDDM phenotype. To obtain more direct evidence for this concept, we studied the effect of long-chain fatty acids (in concentrations similar to those observed in plasma before and after the onset of NIDDM) on the function of cultured islets from control and prediabetic rats. Zhou and Grill (14) have reported that basal insulin secretion at 3 mM glucose is increased severalfold by exposure of normal rat islets for 48 hr to 0.25 mM palmitate

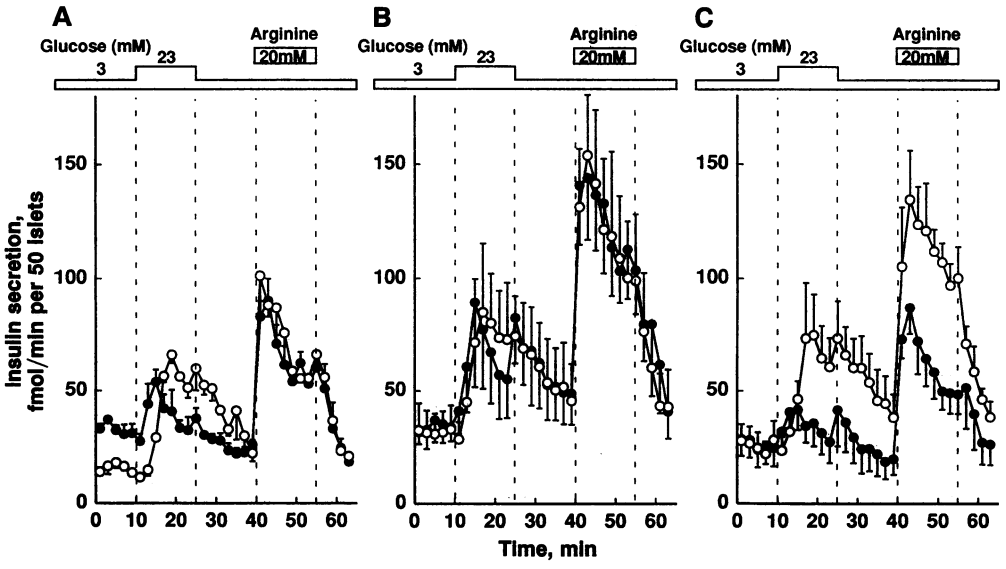

FIG. 2. Insulin secretion by islets perifused with 3 and 23 mM glucose from 6-week-old lean Wistar rats (*A*), obese female (non-prediabetic) ZDF rats (*B*), and obese male (prediabetic) ZDF rats (*C*) cultured for 7 days in 2% BSA alone as a control (○) or in 2 mM FFA mixture with 2% BSA (●). Arginine was perifused at the end of each experiment to exclude insulin depletion as the cause for impairment of GSIS. The incremental insulin response to 23 mM glucose was reduced 68% and 69% in islets of Wistar rats and prediabetic ZDF rats, respectively ($P < 0.05$). Islets of nonprediabetic obese rats were unaffected.

Table 2. Plasma FFA levels (postprandial and after an 8-hr fast) and basal IRI levels in pancreatic effluents of 6-week-old (prediabetic) and 12-week-old obese male ZDF rats and age-matched obese female (nonprediabetic) and lean male ZDF rats.

Age, weeks	Lean ZDF			Obese ♀ ZDF			Obese ♂ ZDF		
	IRI, μU/ml	FFA, mM			FFA, mM			FFA, mM	
		Fed	Fasted	IRI, μU/ml	Fed	Fasted	IRI, μU/ml	Fed	Fasted
6	7.8 ± 1.5	0.41 ± 0.03	1.07 ± 0.14	24.4 ± 3.9	0.57 ± 0.06	1.98 ± 0.05	20 ± 2.0	0.52 ± 0.09	1.09 ± 0.02
12	7.6 ± 0.8	0.90 ± 0.04	1.28 ± 0.20	129 ± 12.6	1.17 ± 0.20	2.44 ± 0.17	142.5 ± 12.5	2.01 ± 0.14	1.70 ± 0.17

The number of FFA determinations ranged from three to six rats. Insulin values represent the mean of five or six perfusion experiments in each group. Each insulin value in a single experiment represents the average of 10 determinations collected at 1-min intervals over a 10-minute period during perfusion at 5.6 mM glucose. All values are means ± SEM.

without BSA, and they also observed a 50% reduction in glucose-stimulated insulin secretion. Since the plasma FFA levels of prediabetic rats ranged from 1.5 to 2.0 mM (Fig. 1*C*), we cultured islets from normal Wistar rats in the presence of a 2 mM 1:2 palmitate/oleate mixture with 2% BSA or 2% BSA alone for 7 days. Perifusion of these islets revealed a 2-fold increase in basal insulin secretion at a glucose concentration of 3 mM ($P < 0.05$) in islets cultured in 2 mM FFAs (32.3 ± 2.2 vs. 18.0 ± 2.4 fmol/min per 50 islets; $P < 0.05$; Fig. 2*A*). This increase was reduced to 24 ± 3.8 fmol/min per 50 islets (not significant) when 2 μM etomoxir, an inhibitor of fatty acid oxidation (30, 31), was present in the culture medium, confirming the findings of Zhou and Grill (14). The 7 days of culture with 2 mM FFA mixture reduced the incremental insulin response to 23 mM glucose from 21.7 ± 0.6 fmol/min per 50 islets to 7 ± 3.9 ($P < 0.05$) in lean rats (Fig. 2*A*) and from 30.1 ± 7.2 fmol/min per 50 islets to 9.4 ± 2.2 ($P < 0.05$) in obese prediabetic rats (Fig. 2*C*). There was no reduction in islets from obese nonprediabetic rats (Fig. 2*B*). This was not the result of insulin depletion, since the post-glucose challenge with 20 mM arginine elicited a brisk response in these islets (Fig. 2*C*). FFAs in the medium did not increase the basal insulin output of islets of obese male and female ZDF rats (Fig. 2 *B* and *C*).

Plasma FFA–Insulin Relationships. That FFA levels in obese prediabetic rats were elevated despite hyperinsulinemia (4) strongly implies an underlying resistance to the antilipolytic action of insulin on adipocytes, as has recently been reported in obese humans (32). As shown in Table 2, the perfused pancreata of 6-week-old obese female rats and the obese male prediabetic rats exhibited a 2- to 3-fold increase in basal insulin secretion compared with lean controls. Although FFA levels of the obese females were close to normal, the high insulin levels in these rats suggest that the hormone was less effective in preventing hyperlipacidemia than in lean animals. In the obese male prediabetic rats, the hyperinsulinemia proved incapable of maintaining FFA levels within a normal range.

DISCUSSION

These results in a rodent model of obesity and insulin resistance provide evidence for a "lipotoxic" cause of some of the β-cell abnormalities observed before and after the onset of NIDDM. First, the onset of progressively increasing hyperlipacidemia, beginning ≈2 weeks before the loss of GSIS and onset of hyperglycemia, provides ample time for the postulated changes in β cells to occur; without exception the plasma FFA concentration exceeded 1.5 mM and islet TG content was greater than 0.7 μg per islet just before the onset of hyperglycemia, both values were significantly greater than those of either control group ($P < 0.001$). (Lipid droplets were detected in sections of diabetic islets.) Second, a moderate reduction of the lipid abnormalities in obese prediabetic rats by means of caloric restriction reduced the β-cell abnormalities of NIDDM, although interpretation of this particular finding is complicated by the multiple consequences of dietary restriction. Third, the first-phase GSIS (at 23 mM glucose) was significantly reduced in islets of lean Wistar and obese prediabetic ZDF rats cultured in a 2.0 mM FFA mixture, but this was not observed in islets from non-prediabetic animals. This suggests a greater vulnerability of prediabetic β cells to high FFA levels. Fourth, a high rate of basal insulin secretion at 3 mM glucose was induced in normal islets cultured in the presence of a 2 mM mixture of long-chain fatty acids; in islets from obese rats, basal insulin secretion was elevated in the absence of FFAs and was not enhanced further by the addition of FFAs to the culture medium. One interpretation of this is that the chronic *in vivo* exposure to higher FFA concentrations had already induced the increased basal secretion of insulin. It should be stressed that, in addition to acute direct stimulation of insulin secretion by long-chain fatty acids (10–14), there is evidence for chronic FFA-induced effects on islets, such as increased numbers of β cells (H. Hirose, L. Inman, and R. H. Unger, unpublished work) and enhanced low-K_m glycolysis (J. Milburn, Y. H. Lee, Y. Nagasawa, A. Ogawa, M.O., H. Beltrandelrio, C. N. Newgard, J.H.J., and R.H.U., unpublished work).

Taken together, these results support a lipotoxic model for the pathogenesis of obesity-related β-cell alterations both before and at the onset of NIDDM. By invoking a relationship in which elevated FFA concentrations concomitantly induce insulin resistance in target tissues and insulin hypersecretion in β cells, one can explain how insulin secretion manages to match the level of insulin resistance and prevent the development of hyperglycemia as obesity progresses. (It is not clear whether the very earliest stage of hyperinsulinemia is FFA-driven.) However, whenever FFA levels in prediabetic rats exceeded 1.5 mM, their β cells apparently became incapable of a further increase in secretory function to parallel the rising insulin resistance; first-phase GSIS is abolished at this stage of the disease (4) and hyperglycemia appears. The results suggest that obesity-related NIDDM is characterized by two defects: (*i*) a primary resistance in adipocytes to the antilipolytic effects of insulin in obese animals, which causes the hyperlipacidemia that, in turn, induces insulin resistance in muscle and insulin hypersecretion and (*ii*) impairment in the β-cell response to glucose.

It remains to be shown that these findings in ZDF rats are relevant to insulin resistance and NIDDM in humans. Hypertriacylglycerolemia and hyperlipacidemia are familiar components of obesity-associated human NIDDM. If hyperlipacidemia has the same pathogenic consequences in human β cells as in rat β cells, the implications of these findings for the prevention and therapy of the disorder, as well as in the search for the genetic basis of the disease, may be far-reaching.

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