Evidence that down-regulation of β -cell glucose transporters in non-insulin-dependent diabetes may be the cause of diabetic hyperglycemia*

L. ORCI[†], M. RAVAZZOLA[†], D. BAETENS[†], L. INMAN^{‡§}, M. AMHERDT[†], R. G. PETERSON[¶],
C. B. NEWGARD^{‡||}, J. H. JOHNSON^{‡§**}, AND R. H. UNGER^{‡§***††}

[†]Center for Diabetes Research, Gifford Laboratories, Departments of ^{**}Internal Medicine and ^{||}Biochemistry, University of Texas Southwestern Medical Center, and [§]Department of Veterans Affairs Medical Center, Dallas, TX 75235; [‡]Department of Morphology, University of Geneva Medical School, Geneva, Switzerland; and [¶]Department of Anatomy, University of Indiana School of Medicine, Indianapolis, IN 46223

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ABSTRACT Non-insulin-dependent diabetes mellitus (NIDDM) is attributed to a failure of pancreatic β cells to maintain insulin secretion at a level sufficient to compensate for underlying insulin resistance. In the ZDF rat, a model of NIDDM that closely resembles the human syndrome, we have previously reported profound underexpression of GLUT-2, the high- K_m facilitative glucose transporter expressed by β cells of normal animals. Here we report that islets of diabetic rats exhibit a marked decrease in the volume density of GLUT-2-positive β cells and a reduction at the electron-microscopic level in the number of GLUT-2-immunoreactive sites per unit of β -cell plasma membrane. The deficiency of GLUT-2 cannot be induced in normal β cells by *in vivo* or *in vitro* exposure to high levels of glucose nor can it be prevented in β cells of prediabetic ZDF rats by elimination of hyperglycemia. We conclude that this dearth of immunodetectable GLUT-2 in NIDDM is not secondary to hyperglycemia and therefore that it may well play a causal role in the development of hyperglycemia.

In various forms of diabetes, loss of glucose-stimulated insulin secretion appears well in advance of impairment in the insulin response to nonglucose stimuli (1–6). We have recently reported that transmembrane glucose transport is deranged early in the course of β -cell decompensation (7) in male Zucker diabetic fatty rats [ZDF/Drt-*fa*(F10)], a partially inbred colony in which obesity and insulin resistance preceded the development of hyperglycemia [only males become diabetic; all females become obese but do not develop diabetes (8)]. As in human non-insulin-dependent diabetes mellitus (NIDDM) patients, the insulin response to glucose disappears prior to impairment in the insulin response to arginine. The loss of glucose responsiveness of β cells is associated with a decrease in glucose uptake by islets and a profound reduction in immunodetectable GLUT-2 (7), the normally expressed high- K_m glucose transporter of β cells (9), and GLUT-2 mRNA (7). The relationships between the loss of GLUT-2, the impairment in glucose-stimulated insulin secretion, and the rise in preterminal plasma glucose concentration were consistent with the proposal that impaired expression of GLUT-2 would impair glucose sensing and cause hyperglycemia (10). However, the parallelism of these abnormalities made it difficult to determine if the hyperglycemia was the cause of the transporter down-regulation or its consequence.

In the present report we provide a complete morphometric analysis of the β -cell defect in GLUT-2 expression at both the light- and electron-microscopic levels and present evidence that the down-regulation is not secondary to hyperglycemia and could therefore be its cause.

METHODS

Pancreata were fixed in Bouin's fluid, embedded in paraffin, and immunostained for insulin and GLUT-2 by the method of Coons *et al.* (11). Consecutive sections (5 μ m thick) were incubated for 2 hr at room temperature with guinea pig anti-insulin antibody (lot 573 1:1000) or with rabbit anti-GLUT-2 antibody (no. 1092 at 1:100) to the C-terminal hexadecapeptide (Arg-Lys-Ala-Thr-Val-Gln-Met-Glu-Phe-Leu-Gly-Ser-Ser-Glu-Thr-Val) predicted from the sequence of the liver transporter (8). The synthetic peptide was coupled to purified protein derivative (PPD) by the method of Lachman *et al.* (12) and was injected with Freund's complete adjuvant subcutaneously into New Zealand White rabbits. Rabbits were boosted after 6 weeks with PPD-coupled peptide suspended in incomplete Freund's adjuvant. Sections were washed with phosphate-buffered saline (PBS; 0.01 M sodium phosphate buffer, pH 7.4/0.15 M NaCl) and exposed for 1 hr to second-layer fluorescein isothiocyanate-conjugated antibody (sheep anti-guinea pig IgG for insulin and goat anti-rabbit IgG for GLUT-2 at a 1:400 dilution). Sections were counterstained with 0.03% Evans blue and examined with the fluorescence microscope. Adsorption of the anti-GLUT-2 antiserum with the immunizing antigen abolished the staining (data not shown). The immunofluorescent staining obtained with the anti-GLUT-2 no. 1092 on normal Wistar rats was similar to that produced by the anti-GLUT-2 antibody used in a previous study (13). Volume density of immunofluorescent cells was determined by the point-counting method (14).

Immunodetection of glucose transporter at the electron-microscopic level was performed by the staphylococcal protein A–gold method (15). The animals were fixed by vascular perfusion with 1% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4. The pancreas was dissected, minced, and processed for Lowicryl embedding at low temperature (16). The same fixation fluid and embedding procedure were used to process the cultured neonatal rat pancreas (17). Thin sections containing islets of Langerhans or cultured islet cells were collected on nickel grids and incubated with affinity-purified anti-GLUT-2 C-terminal hexadecapeptide (1:50) for 2 hr at room temperature, then washed with PBS and exposed to protein A–gold (gold size, $\approx$ 15 nm) for 1 hr. Grids were washed again and counterstained with uranyl acetate and lead citrate before examination in the electron microscope.

Abbreviations: NIDDM, non-insulin-dependent diabetes mellitus; GLUT-2, glucose transporter 2 (high K_m).

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††To whom reprint requests should be addressed at: Center for Diabetes Research, Gifford Laboratories, 5323 Harry Hines Boulevard, Dallas, TX 75235.

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RESULTS

Evaluation of GLUT-2 by Immunofluorescent Staining. To assess quantitatively the glucose transport system of β cells in diabetic rats, GLUT-2 immunostaining of β cells of male ZDF rats was compared morphometrically with that of con-

trol animals (Fig. 1 and Table 1). Sex-matched controls do not exist because, despite comparable obesity and insulin resistance, female ZDF rats never become diabetic (8). Therefore we employed three groups as controls: age-matched nondiabetic female Zucker fatty rats (*fa/fa*), age-matched lean nondiabetic male Zucker heterozygotes (*fa/+*), and age-

FIG. 1. Pairs of consecutive serial sections stained by immunofluorescence (11) with anti-insulin (*Left*) and anti-GLUT-2 (*Right*) antibodies. ($\times 240$.) (A) Islet of normal appearance in a ZDF nondiabetic female. Virtually all insulin-immunofluorescent cells display GLUT-2 immunostaining. In ZDF diabetic male animals (B–D), the islets show a marked reduction (virtual absence in D) of GLUT-2-positive cells. In B and C the islets appear fragmented by tracks of connective tissue. The ratios of GLUT-2-positive cells to insulin-positive cells are shown in Table 1.

Table 1. Comparison of the volume density (Vv) of GLUT-2- and insulin-immunofluorescent cells in islets of nondiabetic male Zucker heterozygotes (A), nondiabetic female ZDF (B), normal male Wistar (C), 12-week-old diabetic male ZDF (D), 35-week-old longstanding diabetic male ZDF (E), and mildly diabetic male ZDF (F) rats*

Rats	Age, weeks	<i>n</i>	Preterminal plasma glucose, mM	GLUT-2 Vv $\times 10^3$	Insulin Vv $\times 10^3$	Ratio GLUT-2 Vv/insulin Vv
A. Nondiabetic lean δ (<i>fa/+</i>)	8–14	9	7.6 $\pm$ 0.2	539.71 $\pm$ 17.81	566.63 $\pm$ 16.12	0.95 $\pm$ 0.01
B. Nondiabetic δ ZDF (<i>fa/fa</i>)	8–15	9	8.2 $\pm$ 0.4	519.69 $\pm$ 27.57	552.20 $\pm$ 22.34	0.94 $\pm$ 0.02
C. Nondiabetic δ Wistar	8–14	5	6.9 $\pm$ 0.3	533.69 $\pm$ 22.50	553.11 $\pm$ 19.07	0.96 $\pm$ 0.01
D. Diabetic δ ZDF (<i>fa/fa</i>)	12	5	29.9 $\pm$ 2.5	106.78 $\pm$ 22.96	432.23 $\pm$ 29.03	0.25 $\pm$ 0.06
E. Diabetic δ ZDF (<i>fa/fa</i>)	35	5	27.4 $\pm$ 2.5	18.60 $\pm$ 4.06	343.63 $\pm$ 24.60	0.05 $\pm$ 0.01
F. Mildly diabetic δ ZDF (<i>fa/fa</i>)	6–8	1	8.0	509.85	531.53	0.96
		1	9.6	351.45	455.47	0.77
		1	10.0	439.78	630.96	0.70
		1	12.4	382.81	623.48	0.61
		1	14.5	314.76	617.04	0.51

Insulin and GLUT-2-expressing cells were quantitated on consecutive paraffin sections of pancreas taken in the juxtasplenic region (18). β cells were recorded positive for GLUT-2 irrespective of the magnitude of the fluorescent signal encircling the cell; see Fig. 1. Five different islets were studied in each pancreas. The formula for determination of insulin volume density (insulin Vv) was modified as previously reported (19) to correct for the overestimation of bright insulin immunofluorescence. Results are mean $\pm$ SEM for *n* animals. Statistical significance of differences was assessed by Student's *t* test. The following pairs of ratio values (last column) were found to be significantly different: E versus A ($P < 0.001$), D versus A ($P < 0.001$), and D versus E ($P < 0.005$).

*Although these data have not been published previously in this form, the values for plasma glucose and the ratio of GLUT-2 Vv/insulin Vv were employed in ref. 7 to demonstrate the negative correlation between the percent of GLUT-2-positive β cells and the level of hyperglycemia.

matched normal Wistar rats. In severely hyperglycemic rats (glucose above 22.2 mM), we observed a profound reduction in GLUT-2 staining of β cells compared to all of the age-

matched nondiabetic control groups (Table 1). In the control groups the ratio of volume density (Vv) of GLUT-2-positive cells to the volume density of insulin-positive cells was

FIG. 2. Electron microscopy comparison of the intensity of GLUT-2 labeling (protein A-gold technique) between nondiabetic lean male *fa/+* (A); nondiabetic female ZDF (*fa/fa*) (B); and diabetic male ZDF (*fa/fa*) (C) rats. ($\times 28,000$.) While A and B show comparable levels of immunogold labeling, a very marked reduction is observed in C. In each panel, an example of a protein A-gold particle associated either with the flat (F) or the microvillar (MV) region of the β -cell plasma membrane has been circled. Not shown here is the fact that total length of plasma membrane occupied by microvilli was reduced in diabetic male ZDF rats (see text). Quantitation in terms of number of immunogold particles per μm length of membrane in each group of animals is presented in Table 2.

Table 2. Protein A–gold labeling of GLUT-2 on the β -cell plasma membrane in nondiabetic male Zucker heterozygote (A), nondiabetic female ZDF (B), male ZDF with mild hyperglycemia (C), and diabetic male ZDF with severe hyperglycemia (D) rats

Rats	Preterminal morning plasma glucose, mM	GLUT-2 density, particles/ μ m		
		Plasma membrane	Flat segments of plasma membrane	Microvillar segments of plasma membrane
A. Nondiabetic lean δ (fa/+)	7.4 $\pm$ 0.2	1.33 $\pm$ 0.7	0.68 $\pm$ 0.05 (731)	2.09 $\pm$ 0.13 (685)
B. Nondiabetic δ (fa/fa)	8.1 $\pm$ 0.4	1.12 $\pm$ 0.09	0.55 $\pm$ 0.05 (780)	1.77 $\pm$ 0.13 (691)
C. Mild diabetic δ (fa/fa)	12.3 $\pm$ 1.3	0.68 $\pm$ 0.06*	0.43 $\pm$ 0.04 [†] (783)	1.07 $\pm$ 0.11* (455)
D. Severe diabetic δ (fa/fa)	24.5 $\pm$ 3.1	0.33 $\pm$ 0.05*	0.24 $\pm$ 0.04* (680)	0.55 $\pm$ 0.08* (297)

Density of immunolabeling is expressed as the number (mean $\pm$ SEM) of gold particles per unit length (1 μ m) of membrane. In each group 54 photomicrographs were examined. Plasma membrane = mean of flat and microvillar segments. In parentheses are the μ m length of plasma membrane evaluated. Data were evaluated on three animals. In each animal three islets from the juxtasplic region of the pancreas (six randomly taken pictures per islet at a calibrated magnification of $\times 34,500$) were used. Background was evaluated on the basolateral plasma membrane of acinar cells: flat membrane, 0.09 $\pm$ 0.02 (320 μ m evaluated); microvilli, 0.12 $\pm$ 0.02 (269 μ m evaluated).

*Value significantly different from that of nondiabetic and diabetic animals ($P < 0.001$).

[†]Value significantly different from that in A ($P < 0.001$) and from that in B ($P < 0.05$).

approximately 1.0; in the 35-week-old diabetic group the ratio of GLUT-2 Vv to insulin Vv averaged only 0.05 $\pm$ 0.01. In younger male ZDF rats with minimal hyperglycemia GLUT-2 was near normal but decreased with the severity of the hyperglycemia (Table 1).

Electron-Microscopic Evaluation of GLUT-2. This profound reduction in the percent of insulin-positive β cells expressing GLUT-2 at the light-microscopic level was corroborated at the electron-microscopic level; the flat and microvillous portions of the plasma membranes of β cells of diabetic rats exhibited, respectively, 64% and 74% reduction in immunodetectable transporter epitope compared to controls (Fig. 2 and Table 2). The decreased number of GLUT-2 immunoreactive sites per unit length of plasma membrane was accompanied by a fall in percent of plasma membrane occupied by microvilli (48% for male nondiabetic versus 37% for mild diabetic and 30% for severe diabetic). Microvilli of β cells contain the highest abundance of GLUT-2 (13).

Effect of Hyperglycemia on GLUT-2. If the loss of immunodetectable GLUT-2 in diabetic rats is secondary to the hyperglycemia, rather than its cause, a high glucose concentration might reduce GLUT-2 immunofluorescence in β cells of normal rats. To test this we studied the effects of high concentrations of glucose *in vivo* on GLUT-2 levels in the β cells of normal Wistar rats; similar *in vitro* studies were carried out in cultured neonatal rat pancreas. For the *in vivo* studies we maintained blood glucose at approximately 11 mM for 7 days by means of a continuous infusion of 50% glucose (20); for the *in vitro* studies glucose concentration in the culture medium was maintained at 33.4 mM for 5 weeks. Immunostainable GLUT-2 of β cells exposed to high glucose concentration did not differ from that of the controls in the *in vivo* (Table 3) and was somewhat increased in the *in vitro* experiments (Table 4). These findings indicate that immunostainable GLUT-2 of β cells of normal rats is not reduced by

Table 3. Comparison of the volume density (Vv) of GLUT-2 and insulin immunofluorescent cells: Islets of normal male Wistar rats infused with either 5% or 50% glucose for 7 days

Rats	Preterminal plasma glucose,* mM	GLUT-2 Vv $\times 10^3$	Insulin Vv $\times 10^3$	Ratio GLUT-2 Vv/insulin Vv
5% glucose	6.4	608.22	608.84	1.00
	6.1	573.43	592.07	0.97
50% glucose	14.7	612.05	637.69	0.96
	10.9	594.49	606.14	0.98

Rats were unrestrained and fully conscious with indwelling catheters employed as described elsewhere (10). Two rats received each treatment.

*Mean value determined on 7 days.

extended periods of exposure to high glucose concentration and are consistent with the report that chronic hyperglycemic clamping in normal rats increases GLUT-2 mRNA in their β cells (10).

Effect of Preventing Hyperglycemia on GLUT-2 Expression in Prediabetic ZDF Rats. The role of hyperglycemia in the GLUT-2 deficiency of β cells in ZDF rats was addressed by feeding male ZDF rats 40 mg of the α -glucosidase inhibitor acarbose (Miles) mixed in their daily diet beginning at 7 weeks of age—at least 1 week before the onset of diabetes. This agent blocks glucose absorption and reduces hyperglycemia (21–23); in contrast to antihyperglycemic agents such as insulin and sulfonylureas, acarbose is not thought to affect β cells. Acarbose-treated male ZDF rats and untreated age-matched ZDF controls were sacrificed at 26 weeks of age. The mean blood glucose level of the former averaged 7.2 $\pm$ 0.28 mM compared with 33 $\pm$ 4.7 mM in untreated controls. In the acarbose-treated group the GLUT-2 Vv/insulin Vv ratio was 0.20 $\pm$ 0.04, significantly below the nondiabetic value ($P < 0.001$) but somewhat higher ($P < 0.02$) than the 0.06 $\pm$ 0.01 value of untreated hyperglycemic control rats. Thus, while prevention of hyperglycemia with acarbose may have increased GLUT-2 expression slightly above the level in untreated hyperglycemic animals, it was still only 20% of normal, suggesting that most of the reduction of immunodetectable GLUT-2 in diabetic ZDF rats occurs independently of and not as a consequence of the hyperglycemia.

GLUT-2 in Other Rat Models of NIDDM. To determine if the reduction in GLUT-2 was unique to this particular colony of ZDF rats with NIDDM or a general finding in other NIDDM models, we conducted preliminary studies in two completely unrelated colonies of rats with NIDDM. One such colony, the diabetic Wistar Kyoto rat, has marked insulin resistance with insulin levels several times higher than the hyperinsulinemic ZDF animals (24); the other, the GK colony, is nonobese and has normal insulin levels, indicating that insulin resistance is minimal or absent; however, glu-

Table 4. Comparison of protein A–gold labeling GLUT-2 density in plasma membranes of monolayer culture of neonatal rat pancreas

Cultured β cells	Glucose, mM	GLUT-2 density, particles/ μ m		
		Plasma membrane	Flat segments of plasma membrane	Microvillar segments of plasma membrane
7 days	5.5	1.04 $\pm$ 0.12	0.36 $\pm$ 0.09	1.32 $\pm$ 0.14
7 days	33.4	1.35 $\pm$ 0.16	0.59 $\pm$ 0.10	2.02 $\pm$ 0.23
5 weeks	33.4	1.59 $\pm$ 0.11	0.80 $\pm$ 0.09	2.42 $\pm$ 0.22

There were 20 photomicrographs in each group. Results are mean $\pm$ SEM.

glucose-stimulated insulin secretion is selectively lost (25). In both groups the V_v ratios of GLUT-2 to insulin were below 0.35. Thus, in three unrelated forms of murine NIDDM, whether insulin resistance and insulinemia is great or minimal, immunostainable GLUT-2 is markedly reduced.

DISCUSSION

The foregoing results reveal in a model of NIDDM a quantifiable histologic abnormality of β cells, a reduction in GLUT-2, that develops in concert with the onset of steady-state hyperglycemia. Whatever its mechanism, the deficit in high- K_m GLUT-2 in diabetic ZDF rats would be expected to impair glycemic discrimination by their β cells as is the case in other β -cell models with GLUT-2 deficiency. For example, GLUT-2 expression is reduced in transformed RIN m5F cells (9) and in islets from normal rats subjected to hypoglycemic clamping for 12 days (10); in both of these models glucose-stimulated insulin secretion is reduced or absent (refs. 26 and 27; I. Komiya and R.H.U., unpublished observations). The relationship between GLUT-2 and glucose-stimulated insulin secretion may be as follows: Normal abundance of the high- K_m glucose transporter ($K_m \approx 15$ –20 mM) provides for rapid equilibration of intracellular glucose concentration of β cells with extracellular glucose above 6 mM; in other words, glucose transport into the β cells is not rate-limiting for glucokinase-catalyzed phosphorylation even at high glucose concentrations. At a normal fasting level of plasma glucose (4–6 mM) the intracellular glucose concentration of β cells does not exceed 6 mM, which is well below the glucose K_m for glucokinase, estimated to be in the 10 mM range. Glucokinase has been postulated to be the sensor for glucose-stimulated insulin secretion (28). As plasma glucose concentrations rise, intracellular glucose of β cells increases proportionately, and the resulting increase in glucose metabolism via glucokinase signals the need for additional insulin release (28). However, on the basis of previously reported rates of transport at physiologic postprandial blood glucose concentrations (29), a reduction in high- K_m GLUT-2 would reduce glucose transport to rates that could limit glucose usage, thereby dampening the insulin response to extracellular glucose.

This deficit of β -cell GLUT-2 may represent the molecular site of the failure of β cells to respond to a rise in blood glucose concentration so as to compensate for insulin resistance. Postprandial hyperglycemia will therefore go uncorrected, causing the round-the-clock hyperglycemia upon which the diagnosis of overt diabetes is made. If the observed reduction in GLUT-2 should prove to be preventable or reversible, it may be possible to restore glucose sensitivity of β cells and improve their capacity to compensate for the insulin resistance that is believed to underlie this disease. In any case the GLUT-2 deficiency constitutes the first histological abnormality detected in β cells of NIDDM of which we are aware. It would not be surprising if this lesion were found to be an early proximal defect in NIDDM of nonrodent species as well.

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