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Expression of GLUT-2 Antisense RNA in β Cells of Transgenic Mice Leads to Diabetes*

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An insulin response to glucose is required to correct hyperglycemia. Two proteins, the glucose transporter GLUT-2 and the glucose-phosphorylating enzyme glucokinase, have been implicated in the control of glucose metabolism in β cells. To study the role of glucose transporter GLUT-2 in the regulation of insulin secretion and in the development of diabetes mellitus, we have obtained transgenic mice expressing high levels of GLUT-2 antisense RNA in β cells. Western blot analysis showed an 80% reduction in GLUT-2 protein in the β cells of these animals. Islets from transgenic mice showed impaired glucose-stimulated insulin secretion. In addition, much higher levels of blood glucose were detected in transgenic mice than in controls when glucose tolerance tests were performed. These results suggest that the reduction of GLUT-2 in the pancreas could be a crucial step in the development of diabetes mellitus.

Glucose homeostasis is maintained within a normal range by the adjustment of glucose production in the liver and glucose uptake by peripheral tissues, mainly skeletal muscle (1). The β cell regulates this balance by secreting insulin so that normoglycemia is established. The main characteristics of non-insulin-dependent diabetes mellitus (NIDDM)[1] are increased glucose production by the liver, together with a lack of glucose uptake by peripheral tissues and a decrease in glucose-stimulated insulin secretion by the pancreatic β cells (1, 2). Although NIDDM is a widespread metabolic disease, its pathogenesis is unknown. Glucose transport and metabolism in the β cell are necessary for both prestored insulin release and insulin synthesis *de novo* (3). High K_m glucose uptake in β cells is originated by glucose transporter GLUT-2 (4). Several reports have indicated that a decrease in GLUT-2 is noted in various animal models of diabetes, which suggests that GLUT-2 is required for normal glucose sensing (5–9). Furthermore, transfection of AtT-20ins cells with the GLUT-2 cDNA confers glucose-stimulated insulin secretion and glucose regulation of insulin biosynthesis (10), and this could not be reproduced after GLUT-1 transfection (11). The glucose-phosphorylating enzyme glucokinase has also been considered to be a crucial step in the control of glucose metabolism in pancreatic β cells (3, 12). Both GLUT-2 and glucokinase have high K_m for glucose, which ensures that the uptake of glucose is proportional to the highest physiological extracellular glucose concentration. It has been suggested that GLUT-2 and glucokinase might work in concert as a "glucose sensing apparatus" that modulates insulin secretion in response to changes in circulating glucose concentrations (1, 13).

The present study was undertaken to investigate the role of a chronic decrease of GLUT-2 in pancreatic β cells in relationship to glucose-induced insulin secretion, using transgenic animals. To decrease functional GLUT-2 glucose transporter in the islets, we have designed a chimeric gene that expresses a GLUT-2 antisense RNA specifically in β cells. Transgenic mice expressing a GLUT-2 antisense RNA showed a high reduction in GLUT-2 protein in β cells, which led to impaired glucose-stimulated insulin secretion, hyperglycemia, and altered glucose tolerance test.

EXPERIMENTAL PROCEDURES

Generation of Transgenic Mice—The general procedures used for microinjection of the RIP-I/anti-GLUT-2 chimeric gene were as described (14). Fertilized mouse eggs were flushed from the oviducts of superovulated C57BL6/SJL mice 6–8 h after ovulation. Male pronuclei of the fertilized eggs were injected with 2 pl of DNA solution (approximately 2 ng/μl), and viable embryos were reimplanted in the oviducts of pseudopregnant mice. The animals were tested for the presence of the transgene by Southern blot of DNA tail samples taken at 3 weeks of age.

Treatment of Animals—Mice were fed *ad libitum* with a standard diet (Panlab, Barcelona, Spain) and kept under a light-dark cycle of 12 h (lights on at 8:00 a.m.). When stated, mice were a fed high carbohydrate diet and water *ad libitum* for 1 week. The high carbohydrate diet was purchased from Nutritional Biochemical Corp., Cleveland, Ohio. This synthetic diet contained 80.5% sucrose, 10.2% casein, 0.3% DL-methionine, 4% cottonseed oil, 2% brewer's yeast, and 2% mineral mix plus 1% vitamin mix.

Blood samples were obtained between 9 and 10 a.m. by decapitation of mice fed a standard or a high carbohydrate diet. Determination of insulin levels in serum samples were made by RIA (CIS, Biointernational, Gif-Sur-Yvette, France). Serum glucose concentration was measured enzymatically (Glucoquant®, Boehringer Mannheim, Germany). The intraperitoneal glucose tolerance test was performed between 10 and 11 a.m. in fed control and transgenic mice. After anesthetizing the mice with avertin, a blood sample was obtained from the tail vein to measure the basal level of glucose by using a Reflotron® autoanalyzer (Boehringer Mannheim). Mice were subsequently administered an intraperitoneal injection of 1 mg of glucose/g of body weight. Blood samples (30 μl) were obtained at different times from the same animals and the levels of glucose determined.

RNA Analysis—Total RNA was obtained from pancreas by the guanidine isothiocyanate method (15). RNA samples (30 μg) were electrophoresed on 1% agarose gel containing 2.2 M formaldehyde. Northern blots were hybridized with an *Eco*RI GLUT-2 riboprobe. A 600-bp *Bam*HI-*Eco*RI fragment of GLUT-2 cDNA was inserted in pBluescript, linearized with *Eco*RV, and transcribed *in vitro* from the T7 promoter, using T7 RNA polymerase. This generated a 650-nucleotide run-off that was used as riboprobe to detect the GLUT-2 antisense RNA from the transgene.

GLUT-2 Protein Analysis—Western blot analysis was performed by standard procedures (16) from total cellular lysates of islets. Islets were

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[1] The abbreviations used are: NIDDM, non-insulin-dependent diabetes mellitus; RIA, radioimmunoassay; bp, base pair(s).

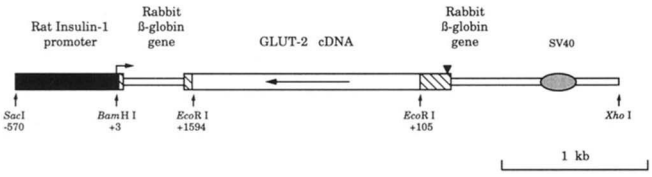

FIG. 1. **Schematic representation of the RIP-I/anti-GLUT-2 chimeric gene used to create transgenic animals.** The *Sac*I/*Bam*HI fragment (−570 bp to +3 bp) of the rat insulin promoter (21) was linked to a *Bam*HI/*Xho*I fragment of the rabbit β-globin gene, which included the two last exons, the last intron, and the 3′ region linked to the SV40 enhancer. The β-globin fragment and SV40 enhancer were included in order to ensure expression of the antisense transgene. An *Eco*RI fragment (+105 bp to +1594 bp) of the GLUT-2 cDNA (19) was introduced in reverse orientation at the *Eco*RI site of the second exon of the β-globin gene. The 4.26-kilobase pair *Sac*I-*Xho*I fragment containing the entire chimeric gene was used to obtain transgenic mice. The *triangle* (▼) represents the polyadenylation signal from the β-globin gene.

disrupted in 5% sodium dodecyl sulfate (SDS), 80 mM Tris-HCl, pH 6.8, 5 mM EDTA, 10% glycerol, and 1 mM phenylmethylsulfonyl fluoride by sonication. Twenty μg of protein was electrophoresed on 9% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. To detect GLUT-2 a rabbit antiserum to GLUT-2 (kindly provided by Dr. B. Thorens), diluted at 1:600, was used.

Insulin Secretion from Islets—Islets were isolated from the pancreas of control and transgenic mice (2 months old) fed a standard diet. Islets were released from pancreatic acinar tissues by digestion with collagenase P (Boehringer Mannheim) (17). Islets were collected by hand-picking under a disection microscope. Batches of six islets were incubated in a shaking water bath for 90 min at 37C in 1 ml of bicarbonate-buffered salt solution containing bovine serum albumin (5 mg/ml, Fraction V, Sigma) supplemented with varying concentrations of glucose (0, 2.8, 5.5, 11.1, or 16.7 mM) or with 10 mM leucine plus 10 mM glutamine. At the beginning of the treatments, vials were gassed with O_2:CO_2 (95%:5%) for 10 min. At the end of the incubation period, supernatants were stored at −20 °C until the insulin was assayed by RIA (CIS, Biointernational, Gif-Sur-Yvette, France). The method allows the determination of 2.5 microunits of insulin/ml, with a coefficient of variation within, and between, assays of 6 and 8%, respectively.

Statistical Analysis—All values are expressed as the means ± 1 S.E. Statistical analysis was carried out using the Student-Newmann-Keuls test, or analysis of variance followed by the Newmann-Keuls test for multiple comparisons, where appropriate (18). Differences were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

To decrease functional GLUT-2 glucose transporter in the islets, we have designed a chimeric gene that expresses a GLUT-2 antisense RNA. It was obtained by linking the rat insulin I promoter (RIP-I) (−570 bp to +3 bp) to an inverted fragment (+105 bp to +1594 bp) of the rat GLUT-2 cDNA (19) (Fig. 1) (RIP-I/anti-GLUT-2). The fragment of rat GLUT-2 cDNA used in this study shares 94% homology with the mouse cDNA (20). The RIP-I promoter directs the expression of chimeric genes specifically in pancreatic β cells (21). Five transmitter-transgenic founder mice were obtained when the RIP-I/anti-GLUT-2 chimeric gene was microinjected into fertilized eggs. These transgenic animals carried from 5 to 25 intact copies of the transgene (data not shown). In the experiments described below we used F1 and F2 mice (2–4 months old) from the IG-1 line, which contained a larger number of copies of the transgene and expressed high levels of GLUT-2 antisense RNA. We also analyzed lines IG-2 and IG-3, which showed similar results. Littermates were used as controls.

Insulin gene expression is induced by glucose (1, 2). Control and transgenic mice were fed a high carbohydrate diet for 1 week to induce the expression of the transgene. Total RNA was obtained from the pancreas and analyzed by Northern blot. High levels of GLUT-2 antisense RNA were detected in the pancreas of transgenic mice fed either a standard or a high carbohydrate diet because of the high expression from the insulin promoter (Fig. 2*A*). No expression of the transgene was detected in other tissues examined, like liver and kidney (data not shown). The concentration of anti-GLUT-2 RNA detected in the pancreas of animals fed a high carbohydrate diet was higher (about 3-fold) than in transgenic mice fed a standard diet. The increase in anti-GLUT-2 RNA led to an 80% decrease in GLUT-2 protein in islets when analyzed by Western blotting (Fig. 2*B*). These results suggested that the expression of the anti-GLUT-2 RNA could block GLUT-2 mRNA, and therefore a lower amount of GLUT-2 protein could be synthesized. Moreover, no differences were detected in the size or the number of islets between control and transgenic mice. The expression of the transgene did not cause islet lesions, even in older mice (data not shown).

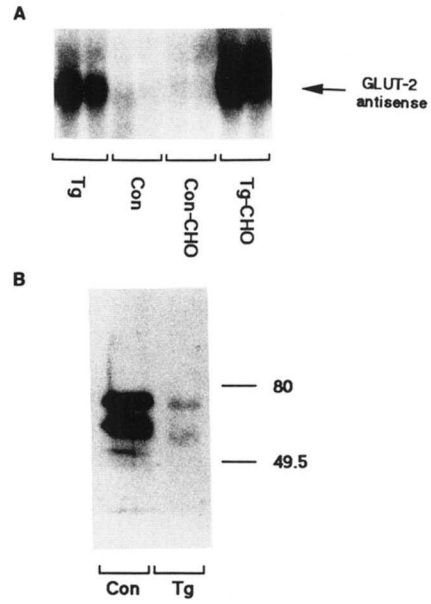

FIG. 2. **Expression of GLUT-2 antisense RNA in pancreatic β cells.** *A*, analysis by Northern blot of GLUT-2 antisense RNA levels. Total RNA was extracted and analyzed as indicated under "Experimental Procedures" from the pancreas of control (*lanes 3–6*) and transgenic (*lanes 1* and *2* and *lanes 7* and *8*) mice fed a standard (*lanes 1–4*) or a high carbohydrate (*lanes 5–8*) diet. *B*, content of GLUT-2 protein in isolated islets. Western blot analysis was performed by standard procedures from total cellular lysates of islets, as indicated under "Experimental Procedures." To detect GLUT-2 a rabbit antiserum to GLUT-2, diluted at 1:600, was used. The appearance of GLUT-2 as a doublet resulted from partial proteolytic degradation of the transporter during the islet isolation procedure before cell lysis. *Lane 1*, control; *lane 2*, transgenic mice.

Fed transgenic mice expressing the RIP/anti-GLUT-2 RNA were hyperglycemic and showed a lower serum concentration of insulin (Table I). In addition, high levels of blood glucose were detected in transgenic mice when intraperitoneal glucose tolerance tests were performed. In contrast to control mice, the glucose levels reached in transgenic animals had not returned to basal values 180 min after the load (Fig. 3). This impaired response to an intraperitoneal glucose tolerance test suggested that transgenic mice developed diabetes (1, 2).

To gain insights into the mechanism by which the reduction of GLUT2 leads to diabetes, we further analyzed insulin secretion in β cells from these animals in response to different nutrient secretagogues. Thus, to discern whether the glucose-stimulated insulin secretion in β cells from the transgenic mice was altered, islets from fed control and transgenic mice were isolated, and the insulin release from different glucose concentrations was measured (Fig. 4). At low glucose concentrations (0 and 2.8 mM), the islets from control and transgenic mice showed similar insulin secretion. However, at the range of glucose con-

TABLE I
Serum concentration of glucose and insulin in fed mice

Serum determinations were made periodically from blood obtained in the morning (9–10 a.m.). Insulin levels were measured by RIA. Glucose was determined enzymatically as indicated under "Experimental Procedures." Results are mean ± S.E. of the animals indicated in parentheses.

	Glucose	Insulin
	mg/dl	*ng/ml*
Control	180 ± 20 (n = 20)	1.71 ± 0.20 (n = 15)
Transgenic	320 ± 28 (n = 20)	1.17 ± 0.18 (n = 18)

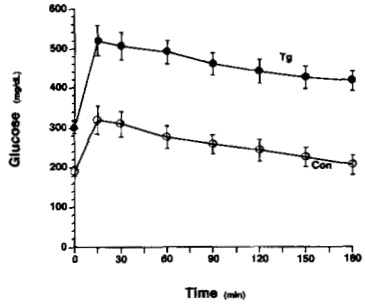

FIG. 3. **Intraperitoneal glucose tolerance test.** Fed transgenic mice had higher basal blood glucose levels than control. These mice were given an intraperitoneal injection of 1 mg of glucose/g of body weight. Blood samples were taken at the times indicated from the tail vein of the same animals as indicated under "Experimental Procedures." Glucose was determined in 30 μl of blood with a Reflotron® (Boehringer Mannheim). Results are mean ± S.E. of 8 transgenic and 6 control mice.

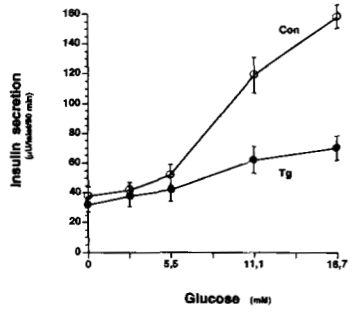

FIG. 4. **Glucose-induced insulin secretion by isolated islets.** Insulin secretion at 0, 2.8, 5.5, 11.1, and 16.7 mM glucose was determined, as indicated under "Experimental Procedures," in islets isolated from control and transgenic mice. Results are the mean ± S.E. of 25 animals in each group.

centrations close to the K_m of GLUT-2 (11.1 and 16.7 mM) (4), an impairment in glucose-stimulated insulin secretion was detected. In contrast, when amino acid-stimulated insulin secretion was studied no decrease was detected in islets from transgenic mice compared to controls. Isolated islets were cultured in 10 mM leucine plus 10 mM glutamine for 90 min, and the insulin levels noted in the incubation medium were: 83.48 ± 15.69 microunits/islet/90 min (n = 16) in islets from control mice, and 108.62 ± 17.95 microunits/islet/90 min (n = 12) in islets from transgenic mice. These results are in agreement with previous observations in other models of spontaneously occurring NIDDM with or without antecedent obesity, which also show lower expression of GLUT-2 in the islets (4).

It has been proposed that the down-regulation of GLUT-2 might cause NIDDM via decreased insulin secretion (4). In models of spontaneously occurring NIDDM with antecedent obesity, such as Zucker diabetic fatty rats (7) and *ob/ob* mice (6, 22), in nonobese rodent models of NIDDM like GK rats (9) or in the neonatal STZ model (6) and in dexamethasone-induced type II diabetes (23), a marked reduction in GLUT-2 expression is observed. Thus, every rodent model of NIDDM shows a defect in glucose-stimulated insulin secretion, together with a reduction in immunostainable GLUT-2 and in high K_m glucose transport function. These findings support the hypothesis that GLUT-2 is required for normal glucose sensing. However, these studies still raise the question as to whether GLUT-2 underexpression is sufficient to cause diabetic hyperglycemia. Our transgenic animal model establishes experimentally that a reduction in GLUT-2 protein leads to a decrease in glucose-stimulated insulin secretion, probably as a consequence of a decrease in glucose transport, since the difference in insulin secretion between islets from control and from transgenic mice is appreciable at glucose concentrations higher than 5 mM. However, these animals did not show any impairment of the amino acid stimulation of insulin secretion, which indicates that the decrease in GLUT-2 in pancreatic β cells may be crucial to the development of diabetes.

We cannot rule out a concomitant alteration on glucokinase activity in the islets of these transgenic mice. Nevertheless, it has to be pointed out that the primary alteration is on GLUT-2. Nevertheless, a defect in the ability of β cells to transport glucose may not be the only explanation of the impairment of insulin secretion by the islets of these mice. The reduction of GLUT-2 may lead to secondary acquired abnormalities in the β cells. A recent study by Hughes *et al.* (11) shows that the role of GLUT-2 in glucose-stimulated insulin release may not be related to the rate of glucose flux and metabolism, but rather might require physical coupling of the transporter with other proteins involved in glucose signaling.

In agreement with our results, which suggest that a primary decrease in functional GLUT-2 levels may lead to diabetes, it has very recently been reported that a mutation in the GLUT-2 gene of a diabetic patient, which results from a single amino acid change (valine 197 to isoleucine) abolished transport activity when the mutant protein was expressed in *Xenopus* oocites (24). Since the patient only had about 50% of the GLUT-2 protein of a non-diabetic individual, this reduction in β cell GLUT-2 protein might have reduced glucose-stimulated insulin release and thus contributed to the development of diabetes in this patient. Then, our results in transgenic mice expressing GLUT-2 antisense RNA in β cells lend support to the hypothesis that defects in GLUT-2 expression may be causally involved in the pathogenesis of non-insulin-dependent diabetes.

Finally, transgenic mice expressing the RIP-I/anti-GLUT-2 chimeric gene provide an experimental system *in vivo* for addressing questions related to β cell function in the absence of the normal expression of GLUT-2.

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