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Overexpression of leptin receptors in pancreatic islets of Zucker diabetic fatty rats restores GLUT-2, glucokinase, and glucose-stimulated insulin secretion

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ABSTRACT **The high-K_m glucose transporter, GLUT-2, and the high-K_m hexokinase of β cells, glucokinase (GK), are required for glucose-stimulated insulin secretion (GSIS). GLUT-2 expression in β cells of Zucker diabetic fatty (ZDF) rats is profoundly reduced at the onset of β-cell dysfunction of diabetes. Because ZDF rats are homozygous for a mutation in their leptin receptor (OB-R) gene and are therefore leptin-insensitive, we expressed the wild-type OB-R gene in diabetic islets by infusing a recombinant adenovirus (AdCMV-OB-Rb) to determine whether this reversed the abnormalities. Leptin induced a rise in phosphorylated STAT3, indicating that the transferred wild-type OB-R was functional. GLUT-2 protein rose 17-fold in AdCMV-OB-Rb-treated ZDF islets without leptin, and leptin caused no further rise. GK protein rose 7-fold without and 12-fold with leptin. Preproinsulin mRNA increased 64% without leptin and rose no further with leptin, but leptin was required to restore GSIS. Clofibrate and 9-*cis*-retinoic acid, the partner ligands for binding to peroxisome proliferator-activator receptor α (PPARα) and retinoid X receptor, up-regulated GLUT-2 expression in islets of normal rats, but not in ZDF rats, in which PPARα is very low. Because the fat content of islets of diabetic ZDF rats remains high unless they are treated with leptin, it appears that restoration of GSIS requires normalization of intracellular nutrient homeostasis, whereas up-regulation of GLUT-2 and GK is leptin-independent, requiring only high expression of OB-Rb.**

The high-K_m glucose transporter, GLUT-2 (facilitative glucose transporter-2) (1) and the high-K_m (high SO.5) hexokinase, glucokinase (GK) (2), are both expressed in normal β cells and are believed to play a role in the regulation of the extracellular glucose concentration and maintenance of euglycemia (3–6). In several rodent models of spontaneous diabetes, Zucker diabetic fatty (ZDF) rats (7), *db/db* mice (8), and BB rats (9), GLUT-2-positive β cells are reduced by at least 40% at the onset of hyperglycemia. Because the GLUT-2 loss coincides temporally with impairment of glucose-stimulated insulin secretion and the onset of hyperglycemia (7, 9), it seemed possible that the reduction in GLUT-2 might be the cause of the β-cell dysfunction and diabetes (5); however, subsequent studies have indicated that the loss of GLUT-2 is secondary to one or more of the metabolic abnormalities of diabetes, and not their cause (8). Of these metabolic abnormalities, hyperglycemia had been exculpated as the down-regulator of GLUT-2 because it increases, rather than decreases, GLUT-2 expression in normal islets (10). Interest has therefore shifted to a second metabolic abnormality of diabetes, high plasma free fatty acid (FFA) levels (11–15). In ZDF rats, plasma FFA levels become elevated approximately 2 weeks before the loss of β-cell GLUT-2 and onset of hyperglycemia (16), raising the possibility that abnormal lipid metabolism is the cause of the loss of GLUT-2. Inasmuch as long-chain FFA have recently been reported to down-regulate GLUT-2, GK, and preproinsulin gene expression in normal rat islets (17), the loss of glucose-stimulated insulin secretion at the onset of hyperglycemia could well be secondary to FFA-induced reduction in β-cell GLUT-2 and/or GK expression.

The adipogenic diabetes of the ZDF rat provides an ideal model in which to study this relationship, because the profound derangement in the lipid metabolism of their islets, the result of a point mutation in their leptin receptor (OB-R) (18, 19), can be reversed by overexpressing the wild-type OB-R gene (20). This makes it possible to determine the role of the lipid abnormalities in the pathogenesis of the β-cell phenotype of adipogenic diabetes.

The aim of this study was to determine whether overexpression of wild-type OB-R would correct the underexpression of key components of the high-K_m glucose metabolism pathway, GLUT-2 and GK, and, if so, whether it was secondary to the normalization of islet lipid metabolism by leptin (21). We therefore repeated our earlier experiments in which the wild-type OB-R gene was transferred into islets of overtly diabetic rats by perfusing an adenoviral construct containing a cDNA encoding the long OB-R isoform into pancreata of ZDF rats (20). We then examined the effects of this on the impaired expression of GLUT-2, GK, and preproinsulin, and on insulin secretion, using as controls islets from pancreata perfused with an adenoviral construct containing the β-galactosidase cDNA. In addition, we measured PDX-1 and CCAAT/enhancer binding protein β (C/EBPβ), two transcription factors known to influence the expression of GLUT-2, GK, and preproinsulin genes (22–25) and to be influenced by fatty acids (17). We also examined the effect on GLUT-2 expression of clofibrate and 9-*cis*-retinoic acid, ligands for the dimerization partners peroxisome proliferator-activated receptor α (PPARα) and retinoid X receptor (RXR) (27), because PPARα is profoundly reduced in ZDF islets (28).

METHODS

Animals. Homozygous (*fa/fa*) ZDF rats were bred in our laboratory from [ZDF/drt-*fa* (F10)] rats purchased from R.

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Abbreviations: OB-R, leptin receptor; GLUT-2, facilitative glucose transporter-2; ZDF, Zucker diabetic fatty rats; FFA, free fatty acids; RXR, retinoid X receptor; C/EBPβ, CCAAT/enhancer binding protein β; PPARα, peroxisome proliferator-activator receptor-α; RT, reverse transcription; GSIS, glucose-stimulated insulin secretion; STAT, signal transducer and activator of transcription.

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Peterson (University of Indiana School of Medicine, Indianapolis). Ten-week-old male diabetic ZDF (*fa/fa*) rats were used for the experiments. Rats were on standard chow (Harlan/Teklad 4% 7001, Teklad, Madison, WI) *ad libitum* and had free access to water. Before each experiment, a blood sample from the tail vein was collected in capillary tubes coated with EDTA. Plasma glucose was measured by using the glucose oxidase method with the Glucose Analyzer II (Beckman). Plasma glucose levels above 200 mg/dl were considered diagnostic of diabetes.

Overexpression of OB-Rb Isoform in ZDF (*fa/fa*) Islets. Pancreata of 10-week-old male diabetic ZDF (*fa/fa*) rats were perfused with 1×10^{12} plaque-forming units (pfu) of recombinant adenovirus containing either the full-length OB-Rb cDNA (AdCMV-OB-Rb) (20) or as a control, the β-galactosidase cDNA (AdCMV-β-gal) (29) in Krebs-Ringer bicarbonate buffer with 4.5% (wt/vol) Dextran T 70, 1% BSA, 5.6 mM glucose, and 5 mM each sodium pyruvate, sodium glutamate, and sodium fumarate. Pancreatic islets were then isolated and maintained for 3 days in suspension culture in 60-mm Petri dishes at 37°C in a humidified atmosphere of 5% CO_2 and 95% air, as described (15). The culture medium consisted of RPMI medium 1640 supplemented with 10% fetal bovine serum, 1% penicillin, 1% streptomycin, and 8 mM glucose. In some experiments 20 ng/ml recombinant leptin was added to islet cultures, (kindly provided by Hector BeltrandelRio and Gayle Yamamoto, Zymogenetics).

Immunoblotting. After 3 days in culture, islets were lysed in SDS/PAGE sample buffer. Total islet protein was quantitated with the bicinchoninic acid assay (Pierce) using BSA as a standard. After resolution of 40 μg of protein (equal loading for each sample) by 7.5% SDS/PAGE, the protein was transferred onto poly(vinylidene difluoride) membranes (Millipore). The membranes were then treated individually with rabbit anti-glucose transporter (GLUT-2) antibody (East Acres Biologicals, Southbridge, MA), anti-glucokinase antibody, anti-PDX-1 antibody (a gift from Chris Wright, Vanderbilt University, Nashville, TN), anti-C/EBPβ antibody (Santa Cruz Biotechnology), anti-STAT3, and anti-phospho-STAT3 (New England Biolabs). After the blots were washed, bound primary antibody was detected by reaction with anti-rabbit IgG antibody-peroxidase conjugate. The antibody complexes were visualized by using a Super Signal-CL Kit (Pierce) as described (20). Signal development times used (10–60 sec) in the detection were within the linear range (data not shown), as were the total amount of proteins used in each immunoblotting (as determined by serial dilution assays; data not shown).

Reverse Transcription–PCR (RT-PCR). Total RNA from 100–200 islets perfused with either AdCMV-OB-Rb or AdCMV-β-gal was extracted by using TRIzol Reagent (Life Technologies, Gaithersburg, MD). After treating with RNase-free DNase I (CLONTECH) reverse transcription was carried out by using 1 μg of total RNA. First-strand cDNA was PCR-amplified with specific oligonucleotide pairs for GLUT-2 (5′-CATTGCTGGAAGAAGCGTATCAG and 5′-GAGACCTTCTGCTCAGTCGACG), GK (5′-GTGGTGCTTTTGAGACCCGTT and 5′-TTCGATGAAGGTGATTTCGCA), PDX-1 (5′-GAGCAGGATTGTGCCGTAACC and 5′-CTCAAAGTTTTCAGAAGCTCG), C/EBPβ (5′-GACAAGCTGAGCGACGAGTAC and 5′-ATTGCATCAAGTCCCGAAACC), and preproinsulin (20). The conditions of PCR were as follows: denaturation for 45 sec at 92°C, annealing for 45 sec at 55°C, and elongation for 1 min at 72°C with either 22 cycles (preproinsulin) or 30 cycles (GLUT-2, GK, PDX-1, and C/EBPβ). The PCR products were subjected to electrophoresis on 1.2% agarose gels and quantified by Southern blot analyses by means of gene-specific ^{32}P-labeled probes for GLUT-2 (5′-GGCCTCAGCTTCATTCTGGGCAATCAGGAT), GK (5′-CAGCAGCTGTACGAGAAGATCATCGGTGGG), PDX-1 (5′-ATCATGAGGCTTAACCTAAACGCCACACAC), C/EBPβ (5′-GCGGAACTTGTTCAAGCAGCTGCCCGAGCC), and preproinsulin (20). As a control for RNA quality and quantity, β-actin mRNA was amplified from all samples by using oligonucleotides as described (30).

Perifusion of Cultured Islets. Islets isolated from ZDF (*fa/fa*) pancreata that had been perfused with AdCMV-

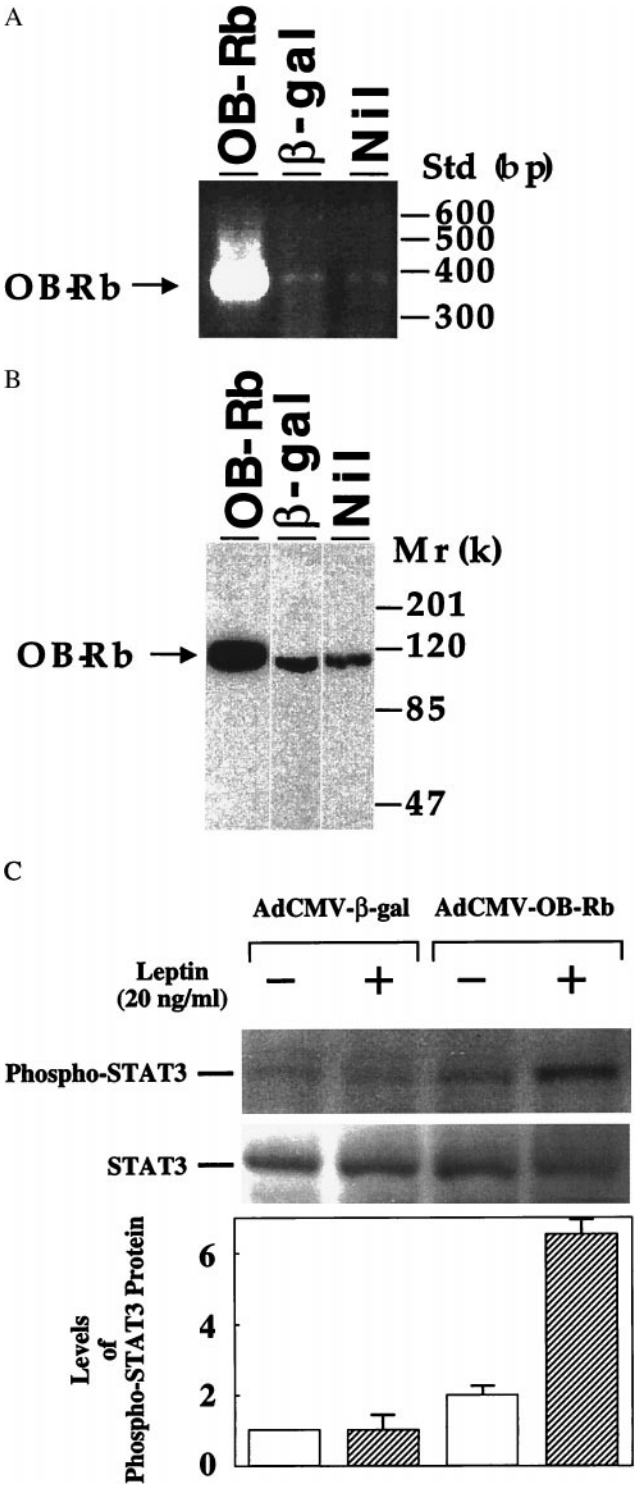

FIG. 1. Effect of pancreatic perfusion of AdCMV-OB-Rb or AdCMV-β-gal on OB-Rb expression and activity in islets of ZDF (*fa/fa*) rats. The rats were 10 weeks old and had diabetes. (*A*) OB-Rb mRNA examined by RT-PCR. (*B*) OB-Rb protein detected by immunoblotting. (*C*) Quantitation of phosphorylated STAT3 measured in OB-Rb-overexpressing islets in the absence and presence of recombinant leptin. Error bars indicate standard deviations ($n = 3$).

OB-Rb or AdCMV-β-gal were cultured for 3 days and then used for perifusion experiments. They were collected under a stereoscopic microscope, washed twice with Krebs-Ringer bicarbonate-Hepes buffer (pH 7.4, 3 mM glucose) and loaded into a 13-mm chamber containing an 8-μm nylon membrane filter (Millipore). Islets were perifused with buffer containing 3 mM glucose, 23 mM glucose, or 20 mM arginine at a flow rate of 0.8 ml/min for 15 min each. Effluent fractions were collected at 2-min intervals, and stored at −20°C until insulin assay. Immunoreactive insulin was determined by radioimmunoassay (31) by charcoal separation.

RESULTS

OB-R Overexpression and Leptin-Mediated STAT3 Activation. Islets from pancreata perfused with AdCMV-OB-R displayed a striking increase in OB-R mRNA (Fig. 1*A*) and OB-R protein (Fig. 1*B*), evidence of successful transfer of the gene to islets. To determine whether the OB-R was functionally active, we measured total and phosphorylated STAT3 in islets from pancreata perfused with AdCMV-OB-Rb or AdCMV-β-gal. Total STAT3 did not change. Phosphorylated STAT3 increased slightly in the AdCMV-OB-Rb-perfused rats in the absence of leptin, but in the presence of leptin, phosphorylated STAT3 increased to 6.5 times the AdCMV-β-gal-infused islet controls (Fig. 1*C*). This finding provided evidence that the receptor was functionally active.

Effects of OB-R Overexpression on GLUT-2 and GK mRNA and Protein Levels. The first β-cell proteins encountered by glucose are GLUT-2, the high-K_m facilitated glucose transporter, and GK, the high-K_m glucose-phosphorylating enzyme. GLUT-2 mRNA and protein, which were extremely low in islets of AdCMV-β-gal-infused controls, were more than 17-fold higher in OB-R-overexpressing ZDF islets in the absence of leptin. The addition of 20 ng/ml leptin had no additional effect on the level of GLUT-2 (Fig. 2 *A* and *B*).

GK mRNA and protein were expressed at very low levels in AdCMV-β-gal-infused control islets. They were, respectively, 7- and 12-fold greater in OB-R-overexpressing islets without leptin, and rose to more than 15 times the control values in the presence of 20 ng/ml leptin (Fig. 2 *C* and *D*).

Effect of OB-R Overexpression with and Without Leptin on Insulin Gene Expression and β-Cell Function. Preproinsulin mRNA was 64% higher in OB-R-overexpressing islets in the absence of leptin and was not further increased by the presence of 20 ng/ml leptin in the culture medium (Fig. 3*A*).

However, in the absence of leptin, glucose-stimulated insulin secretion by OB-R-overexpressing islets was profoundly reduced, as in untreated islets or in islets of AdCMV-β-gal infused controls. Leptin treatment of OB-R-overexpressing

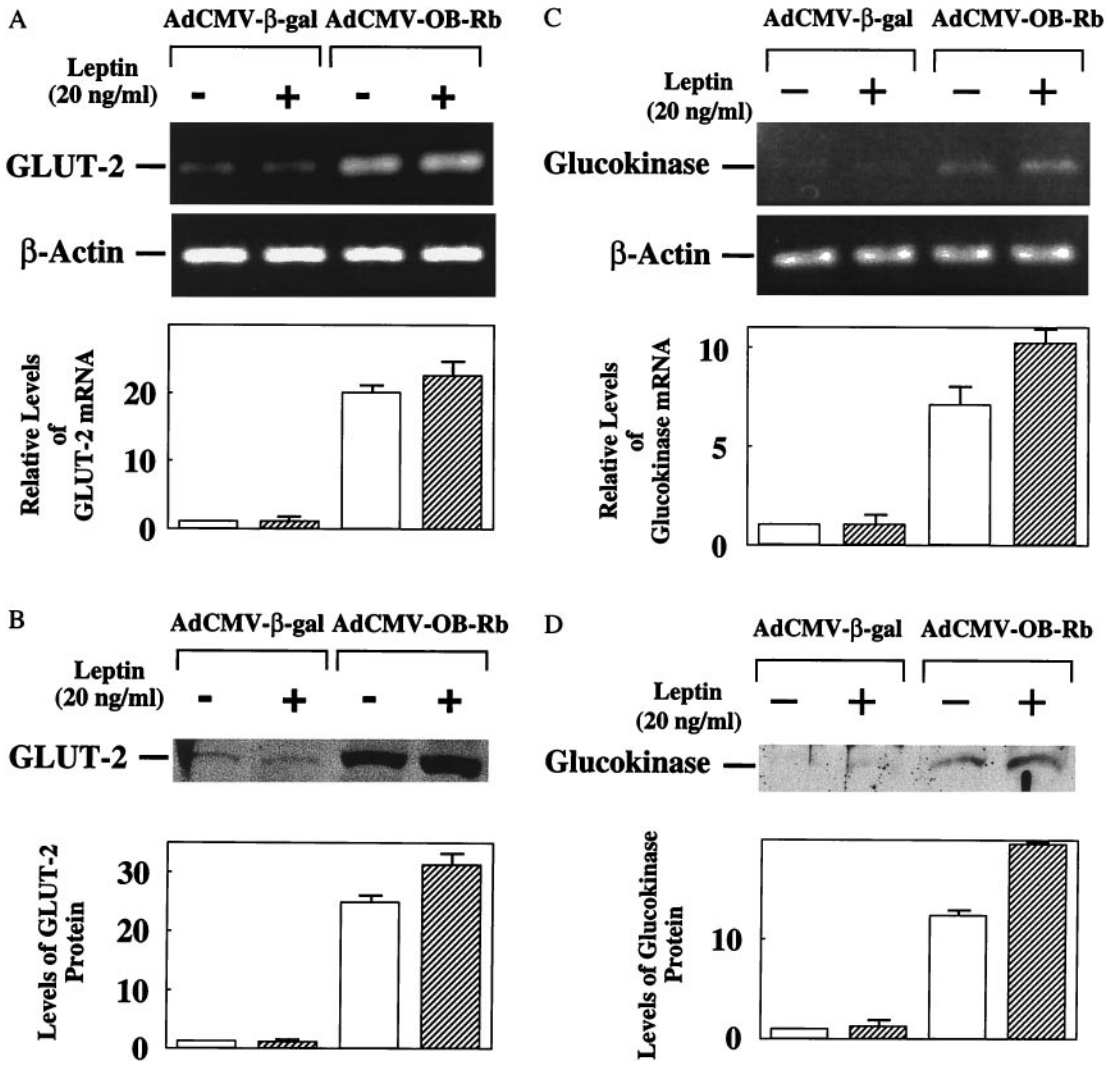

FIG. 2. Effect of perfusion of AdCMV-OB-Rb or AdCMV-β-gal on the mRNA and protein of islet GLUT-2 mRNA semiquantified by RT-PCR and normalized for β-actin mRNA (*A*) and islet GLUT-2 protein quantified by immunoblotting (*B*). (*C*) GK mRNA semiquantitated by RT-PCR and normalized for β-actin mRNA. (*D*) GK protein quantified by immunoblotting. Results from three independent experiments with standard deviations depicted as error bar.

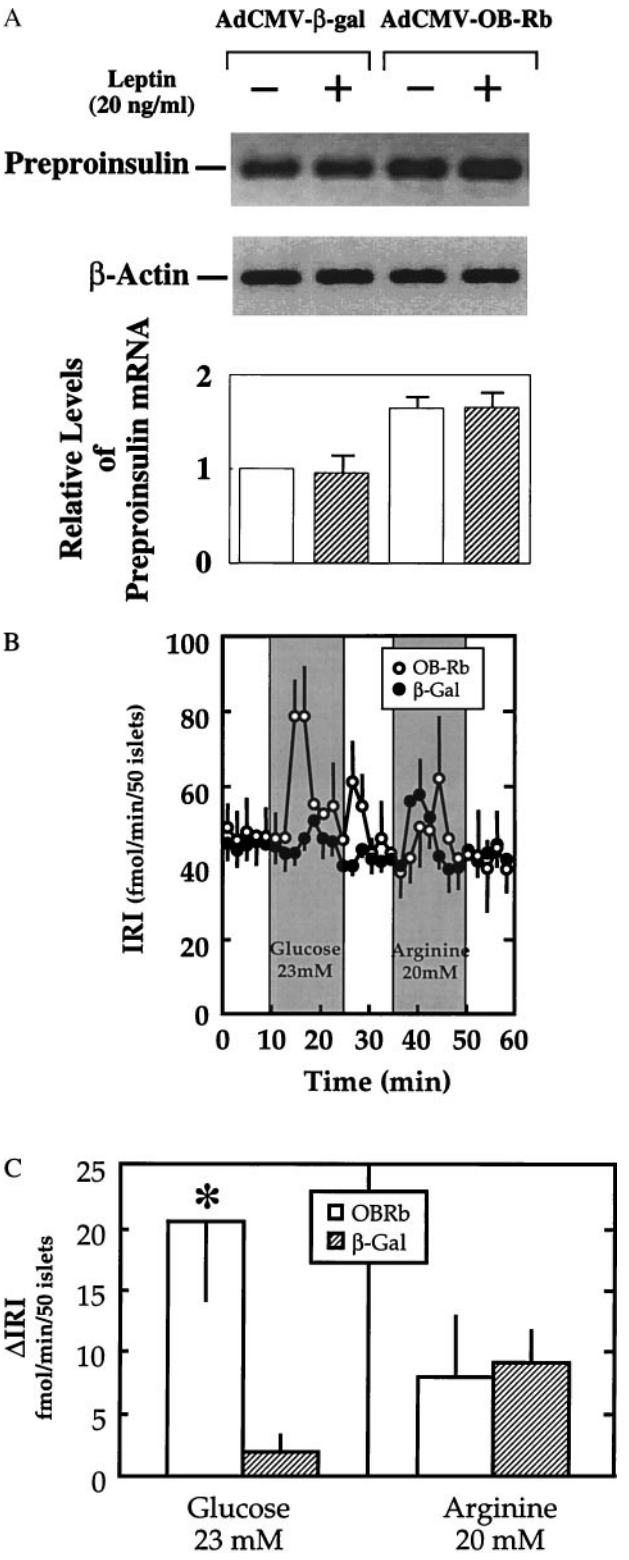

FIG. 3. Effect of perfusion of AdCMV-OB-Rb or AdCMV-β-gal on preproinsulin mRNA semiquantitated by RT-PCR and normalized for β-actin mRNA (*A*) and glucose-stimulated insulin secretion during perifusion (*B*). (*C*) Total insulin production during glucose and arginine stimulation in the experiments in (*B*). Error bars indicate standard deviations ($n = 5$); *, $P < 0.01$ vs. AdCMV-β-gal value.

islets increased the secretory response to 23 mM glucose 8-fold. The response to arginine, which is not impaired in diabetic ZDF islets (7), was not changed by OB-R overexpression (Fig. 3 *B* and *C*).

Effect of OB-R Overexpression on Pancreatic Transcription Factors. The expression of the pancreatic homeodomain transcription factor PDX-1 reportedly can be reduced by long-chain fatty acids (17), which are elevated in ZDF rats (16). C/EBPβ, on the other hand, inhibits transcription of insulin, GLUT-2, and GK genes (24). Because PDX-1 up-regulates these genes, whereas C/EBPβ down-regulates them, changes in those transcription factors could account for the low expression levels of these β-cell genes. We therefore quantified both PDX-1 and C/EBPβ mRNA and protein in AdCMV-OB-R-treated islets and in AdCMV-β-gal-treated controls by RT-PCR and immunoblotting. PDX-1 mRNA increased 2.5-fold, whereas the protein in OB-R-overexpressing islets increased by about 50% in the absence of leptin and by 90% in the presence of leptin (Fig. 4 *A* and *B*). C/EBPβ mRNA decreased by 50%, whereas the protein was 37% lower in OB-R-overexpressing islets without leptin and 51% lower with leptin treatment (Fig. 4 *C* and *D*). The ratio of the PDX-1/C/EBPβ proteins was almost 4 times greater in OB-R-overexpressing islets in the presence of leptin. However, the magnitude of the changes in expression of these two transcription factors was relatively modest compared with the dramatic increases in GLUT-2 and GK mRNA.

Effect of Peroxisome Proliferators on GLUT-2 Expression. We have observed that expression of PPARα is reduced in the islets of obese ZDF rats (28). This reduction raised the possibility that PPARα might be a transcription factor for GLUT-2 in β cells. We therefore examined the effect of clofibrate, a PPARα ligand (32), and 9-*cis*-retinoic acid, the ligand for its dimerization partner, RXR (27), on GLUT-2 mRNA in normal islets. As displayed in Fig. 5, the combination of clofibrate and 9-*cis*-retinoic acid in the culture medium elicited a 2.3-fold increase in GLUT-2 mRNA. This increase suggests that PPARα may also be involved in the expression of genes encoding functionally important proteins in β cells.

DISCUSSION

In these studies, we have overexpressed wild-type OB-Rb (or OB-Rb), the full-length leptin receptor isotype, by adenoviral gene transfer into islets of ZDF (*fa/fa*) rats with mutated OB-R to determine whether the underexpression of GLUT-2 and GK islets of diabetic ZDF rats was secondary to the OB-R mutation and loss of lipopenic action of leptin on islets (21). The transgenic receptor was shown to be functional, because phosphorylated STAT3 (33) was increased slightly in OB-R-overexpressing islets without leptin, but rose far more with leptin. Interestingly, the effects of OB-Rb overexpression on the expression of certain β-cell genes were largely independent of leptin, whereas the effect on insulin secretion was leptin-dependent. For example, our earlier study (20) indicated that the fat content of OB-R-overexpressing islets of ZDF rats remains at high levels unless leptin is added. In other words, lipopenia is quite clearly a ligand-dependent action of OB-R. In the present studies, however, components of the diabetic phenotype relating to gene expression were corrected by OB-R overexpression entirely or largely independent of leptin and of leptin-induced reduction of islet fat. Ligand independence of receptor action has been reported for other receptors when expressed at high levels (34–37), perhaps the result of receptor dimerization. It is therefore possible that at a lower level of OB-R overexpression, ligand independence would not have been observed.

In OB-R-overexpressing islets the low levels of GLUT-2 mRNA and protein were dramatically increased in the absence of leptin and were not further augmented by 20 ng/ml leptin. GK mRNA was 7-fold greater in OB-R overexpressing islets; GK protein, which increased somewhat without leptin, rose further in its presence. Similarly, the baseline level of preproinsulin mRNA was increased independently of leptin. [It

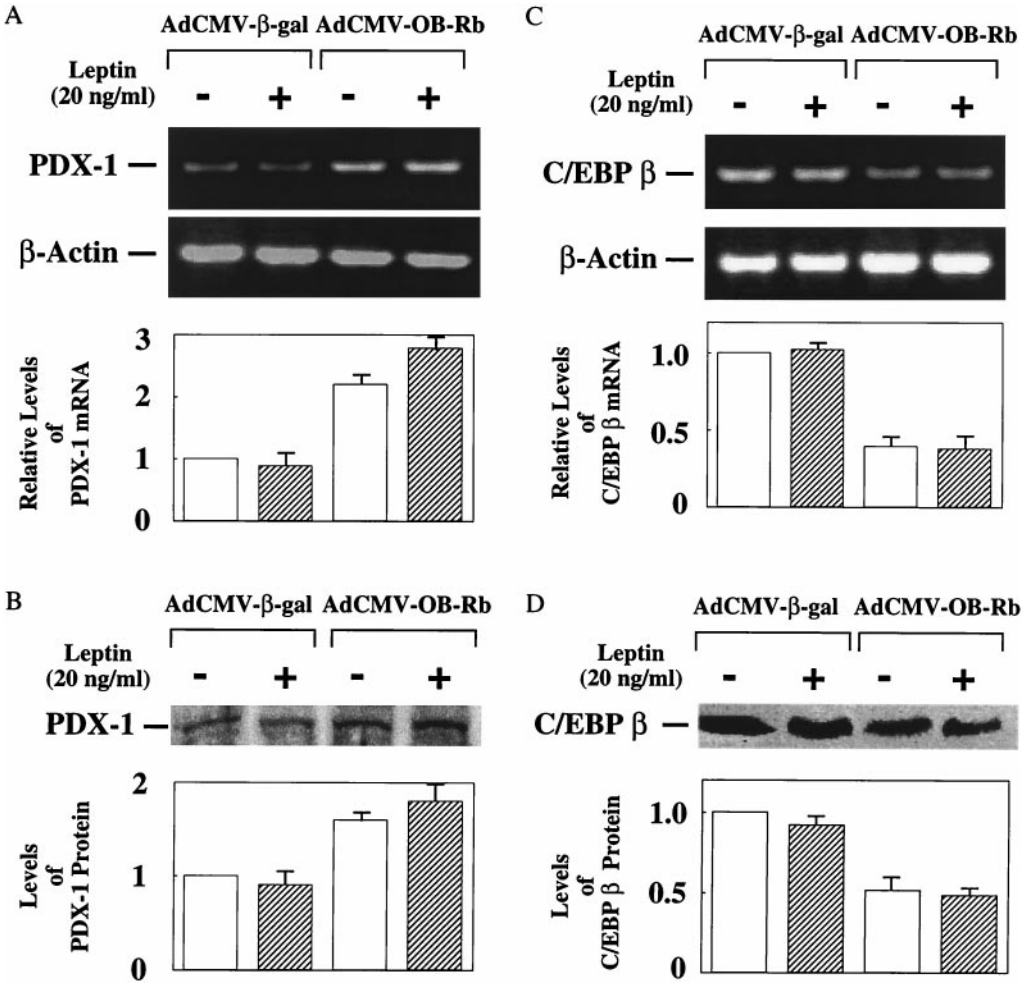

FIG. 4. Effect of perfusion of AdCMV-OB-Rb or AdCMV-β-gal on expression of candidate transcription factors in pancreatic islets. (*A*) PDX-1 mRNA. (*B*) PDX-1 protein. (*C*) C/EBPβ mRNA. (*D*) C/EBPβ protein measured by RT-PCR and immunoblotting. Error bars indicate standard deviations ($n = 4$).

should be noted, however, that when FFA are present in the medium, up-regulation of preproinsulin levels in OB-R-overexpressing islets does require leptin (20)].

By contrast, glucose-stimulated insulin secretion was unchanged by the overexpression of OB-R in the absence of added leptin. When cultured with 20 ng/ml leptin for 2 days, however, islet fat content was reduced (20) in association with an 8-fold increase in glucose-stimulated insulin production (Fig. 3*C*). The insulin response to 20 mM arginine, which is not impaired in the diabetic state (7), was unaffected by OB-R overexpression with or without leptin (Fig. 3*C*). Although leptin greatly reduces the excessive fat content in OB-R-overexpressing islets of diabetic ZDF rats, these studies do not necessarily prove that the improvement in insulin secretion is secondary to the lipopenic action of leptin (21).

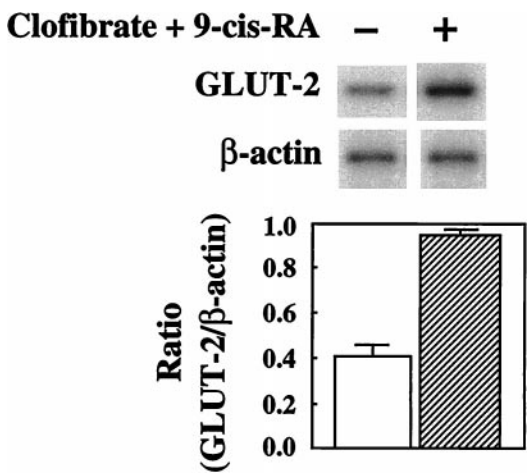

FIG. 5. Effects of clofibrate and 9-*cis*-retinoic acid on GLUT-2 mRNA in normal islets cultured for 48 hr. Error bars indicate standard deviations ($n = 3$).

The mechanism by which the restoration of leptin action in β cells increases (or decreases) the expression of certain genes has not been studied. For example, expression of lipogenic enzymes is reduced by leptin action, whereas expression of enzymes of fatty acid oxidation is increased. The pancreatic homeodomain transcription factor PDX-1 (also known as IDX-1, STF-1, and IPF-1), regulates both the early development of the pancreas and the expression of β-cell genes, including insulin, GLUT-2, and GK (22–24). Long-chain fatty acids reduce PDX-1 and up-regulate C/EBPβ (17), and it has been suggested by others that the underexpression of β-cell genes in ZDF diabetes might be secondary to the reduced PDX-1 and the elevated C/EBPβ (38). It was therefore of interest to assay these proteins in OB-R-overexpressing islets. PDX-1 was higher and C/EBPβ lower in OB-R overexpression. Although the changes were modest compared with GLUT-2, they were in the predicted direction and, like the change in GLUT-2, they were ligand-independent. These transcription factors could be involved in the increase in

GLUT-2 expression that followed the transfer of the OB-Rb gene, but direct evidence for such a role is not provided by this study.

There is now evidence pointing to another transcription factor, PPARα, as a regulator of β-cell gene expression; PPARα expression is reduced in the islets of ZDF *fa/fa* rats, and it is increased independently of leptin by overexpression of wild-type OB-R (28). Here, we report a $\approx$3-fold increase in GLUT-2 mRNA in normal islets cultured with the peroxisome proliferator, clofibrate, an activator of PPARα and 9-*cis*-retinoic acid, the ligand for RXR. This observation suggests that PPARα may be one of the transcription factors involved in the direct up-regulation of GLUT-2 in OB-Rb-overexpressing islets. Consistent with this notion, we note that the promoter and 5′-upstream regions of the genes for both GLUT-2 (39) and GK contain several canonical nuclear receptor binding sites that may serve as RXR/PPARα heterodimer binding sites.

The findings reveal unexpected complexity in the regulation of genes known to be crucial for normal β-cell function. Whereas some of the effects of OB-Rb overexpression, such as improved glucose-stimulated insulin secretion, are leptin-dependent and accompany leptin-mediated lipopenic action, other effects appear to be independent of leptin and its lipopenic action. The former appear to involve secretory functions of β cells, whereas the latter include the enhanced expression of GLUT-2, GK, and preproinsulin genes.

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