Functional and Molecular Defects of Pancreatic Islets in Human Type 2 Diabetes

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To shed further light on the primary alterations of insulin secretion in type 2 diabetes and the possible mechanisms involved, we studied several functional and molecular properties of islets isolated from the pancreata of 13 type 2 diabetic and 13 matched nondiabetic cadaveric organ donors. Glucose-stimulated insulin secretion from type 2 diabetic islets was significantly lower than from control islets, whereas arginine- and glibenclamide-stimulated insulin release was less markedly affected. The defects were accompanied by reduced mRNA expression of GLUT1 and -2 and glucokinase and by diminished glucose oxidation. In addition, AMP-activated protein kinase activation was reduced. Furthermore, the expression of insulin was decreased, and that of pancreatic duodenal homeobox-1 (PDX-1) and forkhead box O1 (Foxo-1) was increased. Nitrotyrosine and 8-hydroxy-2′-deoxyguanosine concentrations, markers of oxidative stress, were significantly higher in type 2 diabetic than control islets, and they were correlated with the degree of glucose-stimulated insulin release impairment. Accordingly, 24-h exposure to glutathione significantly improved glucose-stimulated insulin release and decreased nitrotyrosine concentration, with partial recovery of insulin mRNA expression. These results provide direct evidence that the defects of insulin secretion in type 2 diabetic islets are associated with multiple islet cell alterations. Most importantly, the current study shows that the functional impairment of type 2 diabetic islets can be, at least in part, reversible. In this regard, it is suggested that reducing islet cell oxidative stress is a potential target of human type 2 diabetes therapy. *Diabetes* 54:727–735, 2005

Diabetes is a chronic metabolic syndrome caused by insulin deficiency. There are ~200 million diabetic individuals in the world, with only about half being diagnosed, and these numbers are expected to double by 2030 (1). The disease often results in long-term microvascular, neurological, and macrovascular complications including retinopathy, nephropathy, neuropathy, and increased risk of cardiovascular disease (2). Diabetes is the leading cause of blindness, lower limb amputations, and renal failure in the U.S. The health care cost of diabetes is high, with the total estimated cost in the U.S. exceeding $100 billion (3).

Type 1 and type 2 diabetes are the two main forms of diabetes (4,5). Type 1 diabetes is characterized by an absolute insulin insufficiency caused by the immunological destruction of pancreatic β-cells, which produce and secrete insulin, and it accounts for ~10% of all cases of diabetes. Type 2 diabetes is more complex in etiology and is characterized by a relative insulin deficiency, reduced insulin action, and insulin resistance of glucose transport in skeletal muscle and adipose tissue. The manifestation of frank type 2 diabetes is a continuum of insulin resistance culminating in the failure of augmented insulin secretion to compensate for insulin resistance (6,7), and the progression to full diabetes ensues when pancreatic β-cell hypersecretion of insulin fails to compensate for insulin resistance (7).

Commonly found alterations of insulin secretion in type 2 diabetic patients include reduced or absent first-phase response to intravenous glucose (8), delayed or blunted release after ingestion of mixed meals (9), alterations in rapid pulses, and ultradian oscillations (10). In addition, second-phase insulin secretion and response to nonglucose stimuli may also be reduced (11). Only limited information is available as for the properties of islets isolated from patients with type 2 diabetes. In cells prepared from two type 2 diabetic organ donors, a marked decrease of insulin secretion during glucose stimulation was found, although the secretory response to a combination of leucine and glutamine was less severely affected (12). In addition, the activity of FAD-glycerophosphate dehydrogenase, glutamate-oxalacetate transaminase, or glutamate-pyruvate transaminase was lower in type 2 diabetic islets than in control cells (12). Lin et al. (13) observed that the islets from three type 2 diabetic donors released insulin in pulses with reduced amplitude. More recently, type 2 diabetic islets were shown to have abnormal glucose-induced insulin release during perifusion experiments and reduced efficacy in curing immunodeficient diabetic mice by transplantation (14). In the current study, we prepared isolated islets from the pancreata of 13 type 2 diabetic patients and studied several of their properties, including insulin release in response to glucose and nonglucose stimuli, gene/protein expression of molecules involved in β-cell function, and the concentration of markers

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AMPK, AMP-activated protein kinase; Foxo-1, forkhead box O1; GSH, glutathione; GSSG, GSH disulfide; KRB, Krebs-Ringer bicarbonate solution; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; PDX-1, pancreatic duodenal homeobox-1.

TABLE 1
Main clinical characteristics of type 2 diabetic and nondiabetic donors

Case no.	Age (years)	Sex (M/F)	BMI (kg/m^2)	Cause of death	Cold ischemia time (h)	Known duration of diabetes (years)	PG (mg/dl)	Diabetes treatment
Diabetic donors								
1	72	F	28.8	CVD	12	6	270	Diet
2	75	F	29	Trauma	13	6	417	Sulfonylurea
3	70	F	25.7	CVD	14	5	260	Diet
4	65	F	40.7	CVD	16	ND	398	None
5	77	F	24	CVD	11	3	242	Sulfonylurea
6	69	F	32.7	CVD	10	7	180	Sulfonylurea plus metformin
7	69	M	35	CVD	13	4	239	Diet
8	54	M	28	CVD	15	2	319	Diet
9	49	M	34	CVD	17	ND	244	Diet
10	66	F	25	Trauma	12	4	146	Sulfonylurea
11	69	M	24	CVD	5	23	205	Sulfonylurea plus metformin
12	63	M	29.4	Trauma	15	6	266	Insulin
13	74	M	26	CVD	11	8	134	Metformin
Means ± SD	67 ± 8	—	29.4 ± 5	—	12.6 ± 3.1	5.7 ± 5.7	255 ± 85	—
Nondiabetic donors								
1	62	M	28	Trauma	9	NA	106	NA
2	62	F	25	CVD	12	NA	136	NA
3	47	F	24.6	CVD	11	NA	116	NA
4	61	M	26	CVD	14	NA	103	NA
5	54	F	NA	Trauma	10	NA	98	NA
6	54	M	29.4	CVD	5	NA	104	NA
7	53	M	28	Trauma	12	NA	166	NA
8	74	M	26.2	CVD	13	NA	155	NA
9	76	M	26.3	Trauma	21	NA	159	NA
10	69	M	29.4	CVD	12	NA	136	NA
11	40	M	32	Trauma	10	NA	104	NA
12	69	F	27	CVD	16	NA	63	NA
13	73	M	26.6	CVD	18	NA	161	NA
Means ± SD	62 ± 11	—	27.3 ± 2.1	—	12.5 ± 4.1	—	123 ± 31	—

CVD, cardiovascular disease; NA, not applicable; ND, newly diagnosed; PG, plasma glucose at admission.

of oxidative stress. We found multiple alterations of type 2 diabetic islets, which could be reversed, in part, by reducing oxidative stress.

RESEARCH DESIGN AND METHODS

From July 2001 to June 2004, a total of 147 human pancreata were processed for islet isolation in our laboratory. In this series, there were 13 pancreata from cadaveric donors who were diagnosed as type 2 diabetic patients on the basis of currently accepted criteria (4). The results obtained with the islets from these isolations were compared with those of 13 isolations from nondiabetic donors (Table 1) matched for age, BMI, cause of death, cold ischemia time, collagenase lot, and duration of isolation process. All pancreata studied were obtained and processed with the approval of the ethics committee of our institution. The islets were isolated by enzymatic digestion and density gradient purification, as previously described (15–18). At the end of the isolation procedure, the islets were placed in M199 culture medium containing 5.5 mmol/l glucose, cultured in a CO_2 incubator, and studied within 3–4 days from isolation.

Electron microscopy evaluation. Electron microscopy experiments were accomplished with five control and seven type 2 diabetic islet preparations, as previously described (16,18). Islets were pelletted by centrifugation at 1,300*g* and fixed with 2.5% glutaraldehyde in 0.1 mol/l cacodylate buffer, pH 7.4, for 1 h at 4°C. After rinsing in cacodylate buffer, pellets were postfixed in 1% cacodylate-buffered osmium tetroxide for 2 h at room temperature and then dehydrated in a graded series of ethanol, transferred to propylene oxide, and embedded in Epon-Araldite. Ultrathin sections (60- to 80-nm thick) were cut with a diamond knife, placed on formvar carbon–coated copper grids (200 mesh), and stained with uranyl acetate and lead citrate. Micrographs were obtained at 10,000×. Every microscopic field was analyzed to count β-, α-, and δ-cells, and the respective proportions were expressed as the number of a given endocrine cell type over the total number of endocrine cells × 100.

Insulin secretion studies. Insulin secretion studies were performed by the batch incubation method or using a perifusion procedure, as previously described (15–18). In these experiments (three to five batches at any given experimental condition for each separate islet preparation), after a 45-min preincubation period at 3.3 mmol/l glucose, groups of ~30 islets of comparable size were kept at 37°C for 45 min in Krebs-Ringer bicarbonate solution (KRB) and 0.5% albumin, pH 7.4, containing 3.3 mmol/l glucose. At the end of this period, medium was completely removed and replaced with KRB containing either 3.3 mmol/l glucose, 16.7 mmol/l glucose, 3.3 mmol/l glucose plus 20 mmol/l arginine, or 3.3 mmol/l glucose plus 100 μmol/l glibenclamide. After an additional 45-min incubation, medium samples were collected and stored at −20°C until insulin concentrations were measured by immunoradiometric assay (Pantec Forniture Biomediche, Turin, Italy). In the perifusion studies, batches of ~50 islets were perifused at 37°C (flow rate of 1 ml/min) with 3.3 mmol/l glucose, and then challenged with either 16.7 mmol/l glucose, 3.3 mmol/l glucose plus 20 mmol/l arginine, or 3.3 mmol/l glucose plus 100 μmol/l glibenclamide.

With the islets prepared from four control and four diabetic pancreata, glucose-stimulated insulin secretion studies were also performed after 24-h pre-exposure to 30 μmol/l glutathione (GSH). At the end of these experiments, islets were pelletted and stored at −130°C until measurement of GSH and GSH disulfide (GSSG) was performed by a GSH assay kit (Cayman Chemical, Ann Arbor, MI).

Glucose oxidation experiments. Glucose oxidation was assessed by measuring the formation of $^{14}CO_2$ from [U-^{14}C]glucose, as previously described (19,20). Groups of 15 islets were incubated in a plastic cup in 100 μl of KRB containing 3 μCi D-[U-^{14}C]glucose (specific activity 302 mCi/mmol) plus nonradioactive glucose to a final concentration of either 3.3 or 16.7 mmol/l.

The cups, which were suspended in standard 20-ml glass scintillation vials, were gassed with O_2:CO_2 (95:5%) and were capped airtight. The vials were then shaken continuously at 37°C for 120 min. The islets' metabolism was stopped by injecting 100 μl of 0.05 mmol/l antimycin A (dissolved in 70% ethanol) into the cups. This was immediately followed by an injection of 20 μl hyamine hydroxide (Sigma) into the vials. $^{14}CO_2$ was liberated from the incubation medium by a subsequent injection into the cup of 100 μl 0.4 mmol/l Na_2HPO_4 solution adjusted to a pH of 6.0. After 2 h at room temperature (to allow the liberated $^{14}CO_2$ to be trapped by the hyamine hydroxide), the cup was removed, and 10 ml of a scintillation fluid was added to each vial. Radioactivity was measured in a liquid scintillation counter.

PCR studies. Messenger RNA expression of several genes was measured by real-time quantitative RT-PCR (21). Total RNA was extracted from human pancreatic islets using an RNeasy Protect Mini Kit (Qiagen, Valencia, CA) and quantified by absorbance at A_{260}/A_{280} (ratio >1.65) nm in a Perkin-Elmer spectrophotometer. Integrity was assessed after electrophoresis in 1.0% agarose gels by ethidium bromide staining. cDNA synthesis was performed from 2 μg of total RNA, and the oligonucleotides of interest were obtained from assay-on-demand gene expression products (Applied Biosystem). To avoid amplification of genomic DNA, the primers were designed to span exon-exon borders. The probes were labeled with FAM at the 5′ end and TAMRA at the 3′ end. PCR amplification (40 cycles) was performed in a total volume of 25 μl containing 1 μl cDNA sample, 200 nmol/l of each primer, 100 nmol/l of the corresponding probe, and 12.5 μl of TaqMan Universal PCR Master Mix. Polymerase was activated by preincubation at 95°C for 10 min. The messenger mRNA level of target genes was quantified and normalized for β-actin as previously described (21).

AMP-activated protein kinase protein expression evaluation. The procedure was performed as published previously (22,23) and slightly modified to be applied to human islets. Islet cell lysate aliquots containing 200 μg protein were immunoprecipitated by incubation with total (anti–AMP-activated protein kinase-α [AMPK-α]) and activated (phospho-AMPK-α, Thr172) protein antibodies (Cell Signaling Technologies, Beverly, MA). After immunoprecipitation, bound antibodies were detected using procedures performed according to the manufacturer's instructions (enhanced chemiluminescence; Amersham Biosciences, Buckinghamshire, U.K.). Bands of interest were quantified by a densitometer (GS 690; Bio-Rad, Hercules, CA), using Multi-Analyst/PC-PC Software for image analysis systems, version 1.02 (Bio-Rad).

Determination of islet cell nitrotyrosine concentration. Nitrotyrosine concentration was determined in islet cell lysates by an enzyme-linked immunosorbent assay method as previously described (24,25). White 96-well plates (Iwaki, Chiba, Japan) were coated with 200 μl of standard curve samples (range of concentrations 0.166–15 nmol/l) or 1 μg/μl of islet cell lysates (65 μl/well) in 0.1 mol/l carbonate-bicarbonate buffer (135 μl), pH 9.6, and kept overnight at 4°C. Nonspecific binding was blocked by 1% BSA in PBS plus 0.05% Tween 20 for 1 h at 37°C, and the wells were incubated with purified monoclonal anti-nitrotyrosine mouse IgG (Upstate, Lake Placid, NY) for 1 h at 37°C. Then, the plates were washed and incubated with a peroxidase-conjugated goat anti-mouse IgG secondary antibody for 45 min at 37°C. The peroxidase reaction product was generated using TMB (tetramethyl-benzidine) microwell peroxidase substrate (150 μl/well; Sigma-Aldrich). Plates were incubated 5–10 min at room temperature, and optical density was read at 492 nm in a microplate reader.

Determination of islet cell 8-hydroxy-2′-deoxyguanosine concentration. The 8-hydroxy-2′-deoxyguanosine (8-OHdG) amount was determined in human pancreatic islet DNA digests, using a Bioxytech 8-OHdG-EIA kit, a competitive enzyme-linked immunosorbent assay purchased from OXIS Health Products (Portland, OR) (25,26). All analyses and calibrations (range of 0.5–200 ng/ml) were performed in duplicate. Total DNA was extracted using a Wizard SV Genomic DNA purification system (Promega) and then quantified by absorbance at A_{260}/A_{280} (ratio >1.65) nm in a Perkin-Elmer spectrophotometer. Samples containing 200 μg of DNA were resuspended in 50 μl of reaction mixture containing 100 mmol/l sodium acetate, pH 5.0, and 5 mmol/l $MgCl_2$, and then they were digested with 1 μl DNase I (QIAGEN) for 10 min at room temperature. 8-OHdG monoclonal antibody and the standard or DNA-digested samples were added to a microtiter plate well that had been precoated with 8-OhdG antibody. The optical density was determined with a microplate reader at 450 nm.

Statistical analysis. Results are expressed as the means ± SD. For any given experiment, results from each pancreas were averaged, and *n* indicated the number of pancreata used in that specific setting (see RESULTS). Comparison between groups was performed by two-tailed unpaired Student's *t* test. Unilinear regression analysis was used to correlate insulin secretion and oxidative stress markers.

RESULTS

Electron microscopy. The β-, α-, and δ-cells in the isolated islets were recognized by the typical ultrastructural appearance, and their relative amounts are reported in Fig. 1. The proportion of β-cells was 68.6 ± 3.0 and 61.7 ± 3.7% in control and type 2 diabetic islets, respectively ($P < 0.05$). No statistically significant difference was observed in the percentages of the other islet endocrine cells, although a trend toward increased proportion of α- and δ-cells was found in the diabetic group (Fig. 1).

Insulin secretion. Insulin secretion in response to glucose, arginine, and glibenclamide was studied in control and type 2 diabetic islets, and the results are detailed in Table 2. No significant difference between control and type 2 diabetic islets was observed for insulin release in response to 3.3 mmol/l glucose. However, when challenged with 16.7 mmol/l glucose, diabetic islets secreted significantly less insulin than nondiabetic cells (Table 2). Insulin secretion during arginine and glibenclamide stimulation was also lower from diabetic islets than from control islets (Table 2). However, type 2 diabetic islets released a significantly higher amount of insulin in response to arginine and glibenclamide than in response to glucose (Table 2). Accordingly, when insulin secretion was calculated as the ratio of stimulated release to basal release (stimulation index), the values with diabetic and control islets were, respectively, 1.2 ± 0.4 and 2.2 ± 0.8 in response to glucose ($P < 0.01$), 1.7 ± 0.5 and 2.3 ± 0.4 in response to arginine ($P < 0.01$), and 1.8 ± 0.5 and 2.3 ± 0.7 in response to glibenclamide ($P < 0.05$). Again, a less marked impairment of insulin secretion as induced by nonglucose stimuli in diabetic islets was shown because stimulation indexes of diabetic islets in response to arginine and glibenclamide were significantly higher ($P < 0.01$) than in response to glucose.

In type 2 diabetic patients, a defect of the early phase of insulin release has been consistently reported both in vitro and in vivo (8,14,27). We studied this issue with isolated type 2 diabetic islets, and the results are reported in Fig. 2. Glucose stimulation did not elicit any apparent increase of the early insulin secretion phase from diabetic islets (Fig. 2). However, when challenged with arginine or sulfonylurea, type 2 diabetic islets promptly released insulin, similar to that observed with control islets (Fig. 2).

Intracellular glucose handling. To investigate the possible mechanisms leading to defects in glucose-stimulated insulin secretion, several steps involved in intracellular glucose handling were explored. As shown in Fig. 3, mRNA expression of GLUT1 and -2 and glucokinase was reduced in type 2 diabetic islets, whereas no difference was observed in the expression of phosphofructokinase, aldolase, and pyruvate kinase. In addition, it was found that at 3.3 mmol/l glucose, there was no significant difference between diabetic and control islets in the rate of glucose oxidation (Table 3), whereas at a high exose level, glucose oxidation was significantly lower in diabetic than in nondiabetic cells (Table 3). Thus, the alterations of glucose-induced insulin release from type 2 diabetic islets were accompanied by changes in gene expression of molecules involved in glucose transport and metabolism and by reduced glucose oxidation.

The role of AMPK and of some transcription factors. Because AMPK can modulate several intracellular meta-

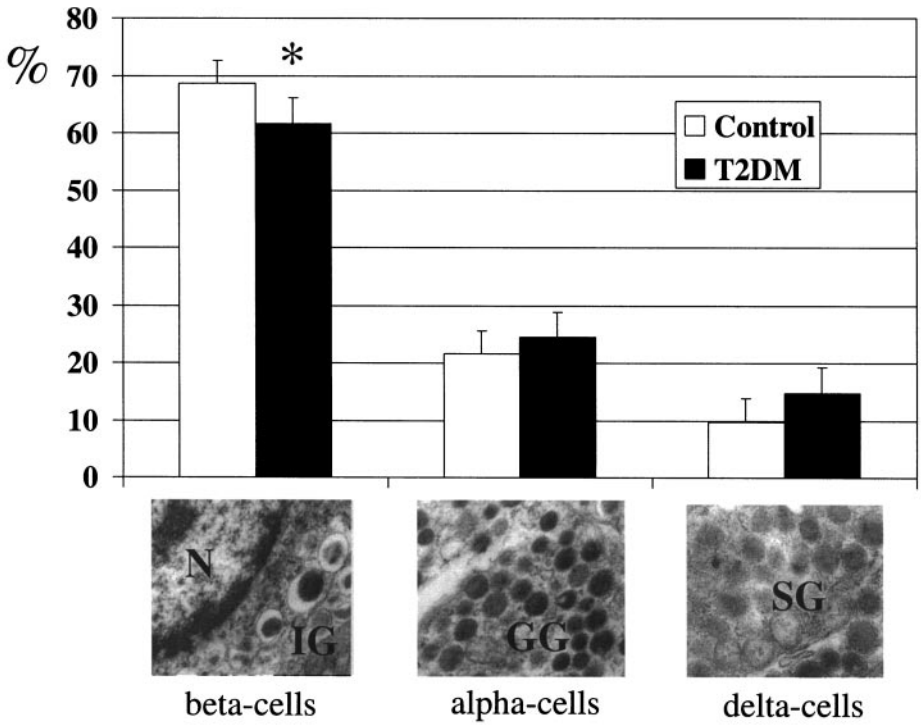

FIG. 1. Endocrine cell composition of control ($n = 5$) and type 2 diabetic (T2DM; $n = 7$) islets. Bars represent percentage values, and the bottom microphotographs are representative images of β-, α-, and δ-cells, recognized by their characteristic granules on electron microscopy examination. N, nucleus; IG, insulin granules; GG, glucagon granules; SG, somatostatin granules. *$P < 0.05$ vs. control islets.

bolic pathways and may have a role in the regulation of insulin secretion (23,28), its protein expression and activation was evaluated in control and diabetic islets. As shown in Fig. 4, total protein expression was not different in nondiabetic versus diabetic islet cells. However, the percentage of activated protein was significantly lower in type 2 diabetic than control islets (Fig. 4), so that the ratio of activated to nonactivated protein was ~30% lower in the former.

It is known that several transcription factors affect β-cell function (29,30). In the current study mRNA expression of pancreatic duodenal homeobox-1 (PDX-1) and forkhead box O1 (Foxo-1) were measured, and it was found that both of these factors were expressed more in diabetic than in nondiabetic islets (Fig. 5). This was associated with decreased insulin expression in type 2 diabetic islet cells (Fig. 5).

The role of oxidative stress and the effects of GSH. Because increased oxidative stress has been reported to be associated with the diabetic condition in several experimental and clinical settings (24,31,32), we measured the concentration of nitrotyrosine and 8-OHdG, markers of oxidative stress, directly in the islets. Both parameters were significantly higher in diabetic islets (10.1 ± 1.1 nmol/l and 9.7 ± 2.5 ng/ml, respectively) than in control cells (5.8 ± 0.5 nmol/l and 1.9 ± 0.4 ng/ml, respectively) (Fig. 6), and the concentration of nitrotyrosine as well as that of 8-OHdG were inversely and significantly correlated with insulin secretion (Fig. 7). According to these findings, when type 2 diabetic islets were pre-exposed to the antioxidant agent GSH, a significant improvement of glucose-stimulated insulin secretion was observed, with normalization of the stimulation index (Fig. 8*A*). This was associated with reduced nitrotyrosine concentration, which decreased to 5.8 ± 1.6 nmol/l (Fig. 8*B*), and partial restoration of insulin mRNA expression (Fig. 8*C*). In nondiabetic islets, no significant change after GSH culture was observed for insulin secretion (stimulation index from 2.1 ± 0.6 to 2.2 ± 0.7) and nitrotyrosine level (from 5.8 ± 0.4 to 5.7 ± 0.5 nmol/l). When GSH and GSSG concentrations were measured, the values in control islets were 4.7 ± 0.5 and 2.3 ± 0.6 μmol/mg protein, respectively, with a ratio of 2.1 ± 0.3. In type 2 diabetic islets, lower levels of GSH (3.3 ± 0.7 μmol/mg protein) and higher levels of GSSG (4.0 ± 0.4 μmol/mg protein, $P < 0.05$ vs. control islets) were found, with the resulting GSH-to-GSSG ratio significantly lower than in the nondiabetic cells (0.8 ±

TABLE 2
Insulin secretion results ($\mu U \cdot islet^{-1} \cdot 45\ min^{-1}$) from control and type 2 diabetic islets in response to 3.3 mmol/l glucose, 16.7 mmol/l glucose, 3.3 mmol/l glucose plus 20 mmol/l arginine, or 3.3 mmol/l glucose plus 100 μmol/l glibenclamide

	3.3 mmol/l glucose	16.7 mmol/l glucose	3.3 mmol/l glucose plus 20 mmol/l arginine	3.3 mmol/l glucose plus 100 μmol/l glibenclamide
Control islets	2.47 ± 0.45 (13)	5.40 ± 0.90 (13)*	5.53 ± 0.62 (11)*	5.63 ± 0.69 (11)*
Type 2 diabetic islets	2.10 ± 0.61 (13)	2.50 ± 0.60 (13)‡	3.45 ± 0.72 (10)†‡	3.71 ± 0.75 (10)†‡

Numbers of pancreata studied are given in parentheses. *$P < 0.01$ vs. controls at 3.3 mmol/l glucose; †$P < 0.05$ vs. type 2 diabetic islets at 3.3 and 16.7 mmol/l glucose; ‡$P < 0.05$ vs. the respective values of controls.

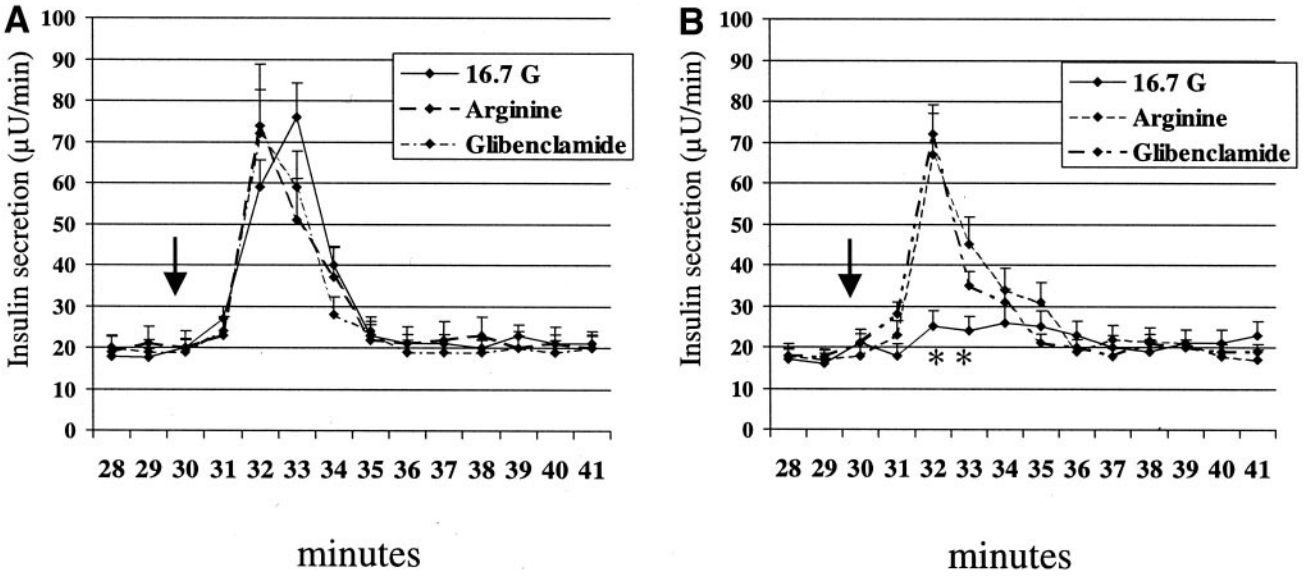

FIG. 2. Insulin secretion from type 2 diabetic islets during perifusion experiments. For these experiments, ~50 islets from five separate preparations were placed in a perifusion chamber and perifused with 3.3 mmol/l glucose solution for 30 min, and then (arrow) with either 16.7 mmol/l glucose (16.7 G), 3.3 mmol/l glucose plus 20 mmol/l arginine (Arginine), or 3.3 mmol/l glucose plus 100 μmol/l glibenclamide (Glibenclamide). The flow rate was 1 ml/min, and samples were collected every minute. Compared with control islets (*A*), type 2 diabetic islets (*B*) did not show any apparent increase of early insulin secretion during 16.7 mmol/l glucose, whereas 3.3 mmol/l glucose plus 20 mmol/l arginine and 3.3 mmol/l glucose plus 100 μmol/l glibenclamide elicited a peak of insulin release. *$P < 0.05$ of 16.7 mmol/l glucose vs. the other experimental conditions.

0.3, $P < 0.05$). Pre-exposure of type 2 diabetic islets to GHS normalized these parameters (GSH: 4.2 ± 0.6 μmol/mg protein; GSSG: 2.1 ± 0.4 μmol/mg protein; GSH-to-GSSG ratio: 2.1 ± 0.6).

DISCUSSION

In the current study, several defects of pancreatic islets isolated from type 2 diabetic subjects were described. Because the percentage of β-cells was only slightly (although significantly) reduced in diabetic islets as compared with control islets (−10%), we are inclined to believe that the alterations we found were not merely caused by a defect of β-cell mass, but that they mostly reflected primary functional and molecular alterations of the diabetic β-cells.

We confirm that type 2 diabetic islets release less insulin than control islets in response to glucose (12,14), and we show, for the first time, that this is accompanied by altered expression of glucotransporters (in particular GLUT2) and of glucokinase. GLUT2 has high transport capacity and K_m for glucose, so that the rate of transport of this exose by GLUT2 is largely dependent on the ambient glucose concentration (33). On the other hand, glucokinase (hexokinase IV) exhibits low affinity for glucose, and it is considered the rate-limiting step for glucose metabolism in the β-cell (34,35). Alterations of both proteins (and in particular of glucokinase activity) have been associated with diabetes (36). In our study, we did not perform experiments to evaluate glucose transport and phosphorylation in the isolated islets. Therefore, it is not possible to directly infer the relationship between kinetic data of glucose transport/phosphorylation and subsequent functional effects. However, it is of some interest that in type 2 diabetic islets the 40–50% decrease of GLUT2 and glucokinase mRNA expression correlated well with the observed 50% reduction of glucose oxidation.

We found that the defect of insulin release upon nonglucose stimuli was less markedly affected, which is in agreement with previous studies showing that amino acid–induced insulin secretion is better maintained in type 2 diabetic islets (12). In addition, we observed that both arginine and glibenclamide induced an early insulin secretion phase from the diabetic islets. Both arginine (by in-

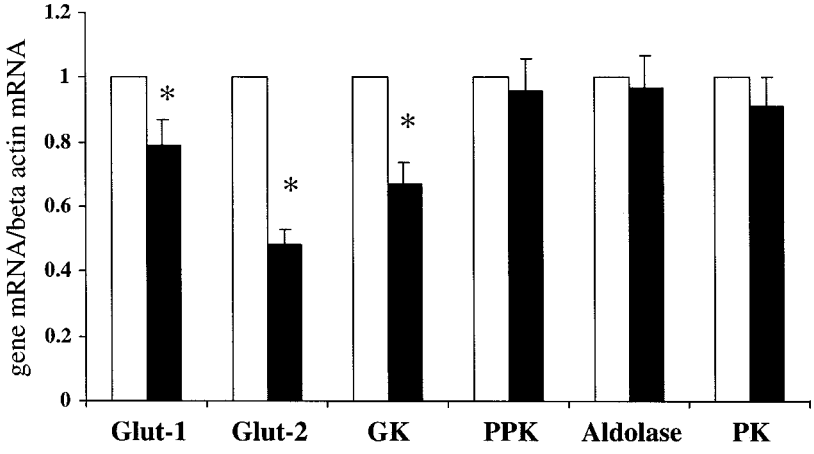

FIG. 3. Quantitative PCR experiments to show mRNA expression of GLUT1 and -2, glucokinase (GK), phosphofructokinase (PPK), aldolase, and pyruvate kinase (PK) in control and type 2 diabetic islets. Bars represent mean and standard deviation values of eight separate experiments with either control or diabetic islets. *$P < 0.05$ vs. control. □, control; ■, type 2 diabetic.

TABLE 3
Glucose oxidation (pmol/l · islet^{-1} · 120 min^{-1}) in control and type 2 diabetic islets (5 separate preparations each)

	3.3 mmol/l glucose	16.7 mmol/l glucose
Control islets	15.9 ± 6.7	42.7 ± 9.6*
Type 2 diabetic islets	12.1 ± 6.1	21.0 ± 5.9†

*$P < 0.05$ vs. controls at 3.3 mmol/l glucose; †$P < 0.05$ vs. controls at 16.7 mmol/l glucose and type 2 diabetic islets at 3.3 mmol/l glucose.

creasing intracellular positive charge) and sulfonylureas (by inhibiting K^+ loss from β-cell) cause depolarization of β-cell membrane (37). This event is followed by Ca^{2+} entry, increased cytosolic Ca^{2+} concentration, and translocation and exocytosis of insulin granules (37). Altogether, these findings underline the importance of searching for selective β-cell defects in the pathways leading from glucose transport to exocytosis of the insulin granules. Based on the available evidence (12, the current study) we are inclined to believe that the defects of glucose-stimulated insulin release in type 2 diabetic β-cells lie between glucose transport and depolarization of the cellular membrane. In this regard, alteration of mitochondrial function might play a crucial role, as recently suggested (38).

A novel finding of the current report is the reduced activation of AMPK that was observed in type 2 diabetic islets compared with control islets. AMPK is a multisubstrate kinase regulated by AMP, and it controls a number of metabolic enzymes with the aim of reducing cellular ATP consumption during conditions of metabolic stress (28). Activation of AMPK is considered to have beneficial effects on diabetes because it improves insulin action in the muscle and liver (28). This kinase has been recently implicated in the control of insulin secretion. Some researchers have reported that increased activity of AMPK leads to inhibition of glucose-stimulated insulin release in MIN6 cells and rodent islets, whereas in other studies activation of AMPK potentiated glucose-induced insulin secretion from isolated rat islets or perfused pancreata (22,39). Because in nondiabetic human islets activation of AMPK by AICAR (5-imidazolecarboxamide riboside) induces a potentiation of insulin release (R. Lupi, unpublished observations), we are inclined to believe that the

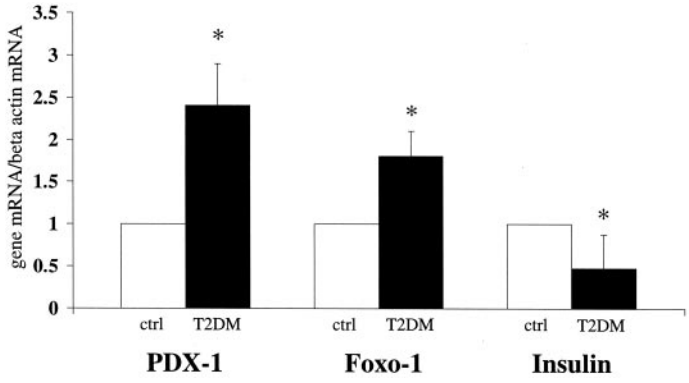

FIG. 5. Quantitative PCR experiments to show mRNA expression of PDX-1, Foxo-1, and insulin in control (ctrl) and type 2 diabetic (T2DM) islets. Bars represent mean and standard deviation values of eight separate experiments with either control or diabetic islets. *$P < 0.01$ vs. control.

reduced amount of activated AMPK that we observed in type 2 diabetic islets may contribute to the defect of insulin secretion.

For the first time, we report that some transcription factors regulating β-cell differentiation and function are altered in type 2 diabetic islets. PDX-1 has a crucial role in early organogenesis and is also a key regulator of differentiated β-cells (29,30,40). This factor supports insulin gene transcription and activates a large number of genes involved in normal β-cell function and survival. We found that in type 2 diabetic isolated islets, mRNA expression of PDX-1 was increased, despite the augmented expression of Foxo-1, which is considered an inhibitor of PDX-1 gene expression (29,30). The increased PDX-1 mRNA expression may be interpreted as an attempt to compensate for the reduced β-cell function of type 2 diabetic islets. However, the effects of this effort remain elusive because gene transcription of key proteins of β-cell function (such as GLUTs and insulin, as observed in our experiments) remained lower than that observed in control islets. Our findings on the increased PDX-1 mRNA expression in type 2 diabetic islets are at odds with data showing that PDX-1 expression is reduced in animal models of diabetes (31). In addition, it has been reported that chronic oxidative stress may decrease PDX-1 transcription in cell line experiments

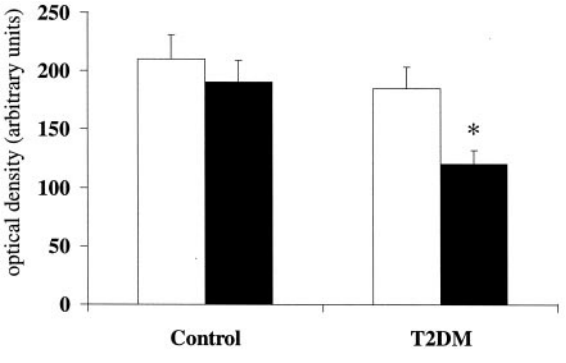

FIG. 4. Immunoblotting experiments to evaluate total and activated protein expression of AMPK in control and type 2 diabetic (T2DM) islets. Bars represent mean and standard deviation values of five separate experiments with either control or diabetic islets. *$P < 0.05$ vs. activated AMPK in control islets. □, total AMPK; ■, activated AMPK.

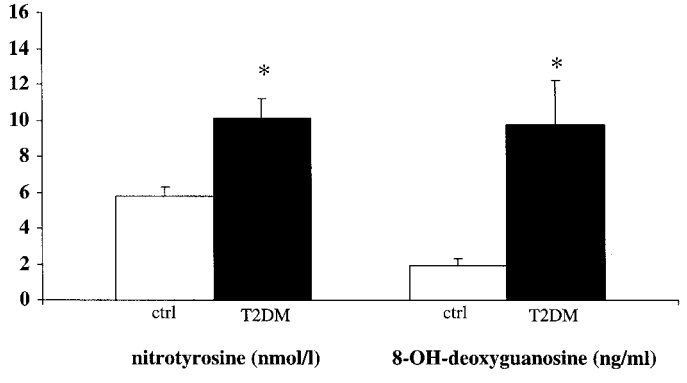

FIG. 6. Nitrotyrosine and 8-OHdG concentrations in control (ctrl) and type 2 diabetic (T2DM) islets. Bars represent mean and standard deviation values of 13 separate experiments with either control or diabetic islets. *$P < 0.01$ vs. control.

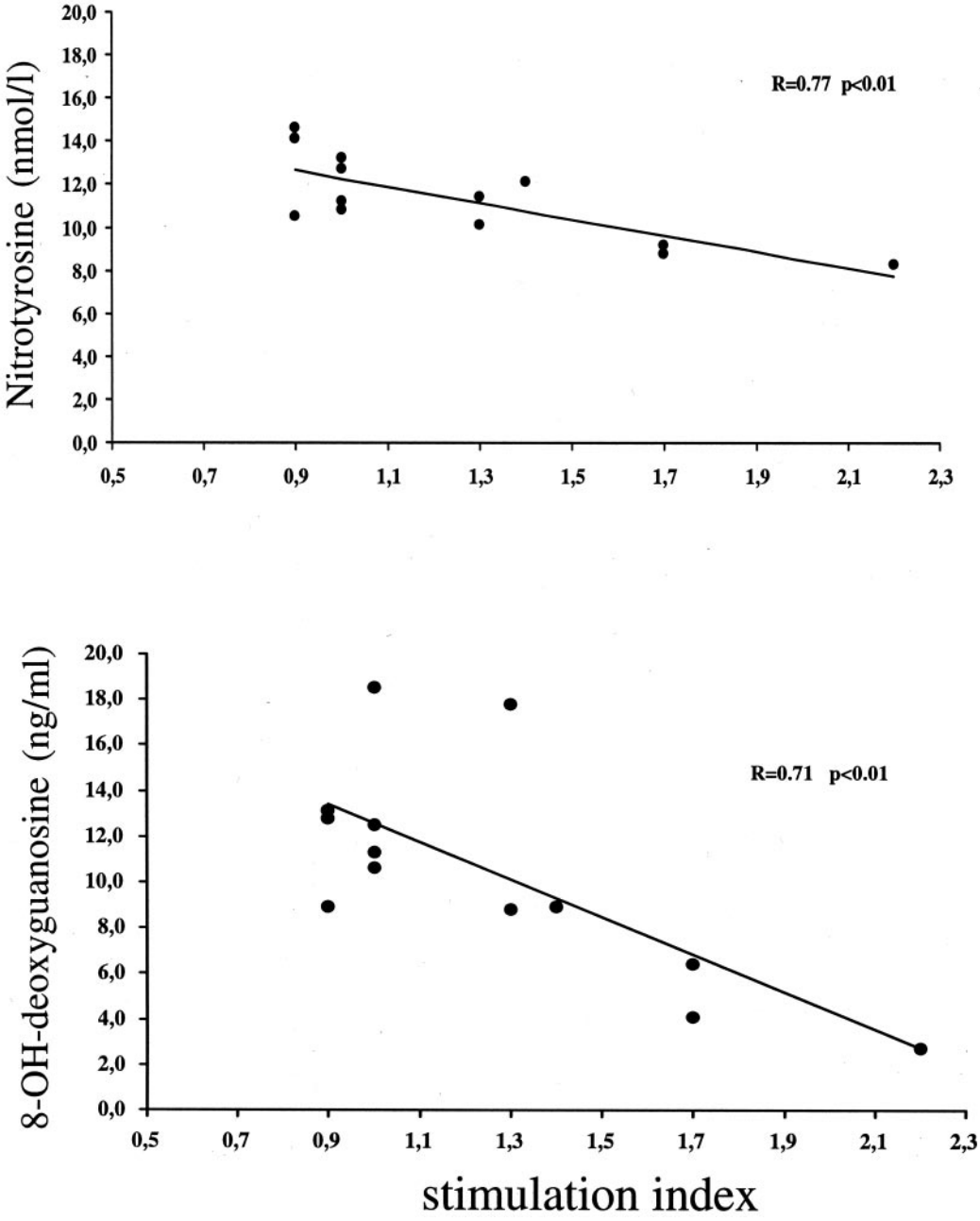

FIG. 7. Correlation of nitrotyrosine and 8-OHdG concentrations with stimulation index of type 2 diabetic islets, showing that when oxidative markers increase, glucose-stimulated insulin secretion decreases.

(41). This controversial issue will therefore require further studies to better understand the complex network of signals and regulatory factors driving PDX-1 regulation and function in type 2 diabetes.

Many studies suggest that type 2 diabetic patients are subjected to chronic oxidative stress (24,31,32). Hyperglycemia and increased free fatty acid concentration can contribute to the formation of reactive oxygen species, which in turn damage cell proteins and DNA. In islet β-cells, oxidative stress is even more dangerous than in other tissues because of the low intrinsic antioxidant capacity of the β-cell (41,42). In our experiments, we found that levels of nitrotyrosine and 8-OHdG, which are markers of oxidative stress, are higher in type 2 diabetes than in control islets, and for the first time we demonstrate a correlation between the concentration of these compounds and the degree of insulin secretion impairment. Notably, the addition of GSH in the incubation medium determined reduction of oxidative stress (as suggested by the diminished levels of nitrotyrosine), improved glucose-stimulated insulin secretion, and increased insulin mRNA expression. These novel findings support the observation by Paolisso et al. (43) that in patients with impaired glucose tolerance, GSH infusion ameliorated insulin secretion, and they add value to the numerous experimental data demonstrating that potentiating β-cell antioxidant defenses is a means to protect the islet cells from oxidative stress (24,31,32,41, 42). Most importantly, our findings demonstrate that the defects of glucose-stimulated insulin release of type 2 diabetic islets are reversible, at least in part. In the current study we did not perform experiments to evaluate the in vivo function of type 2 diabetic islets. A previous report by Deng et al. (14) has clearly shown that, as compared with control islets, islets prepared from diabetic subjects were

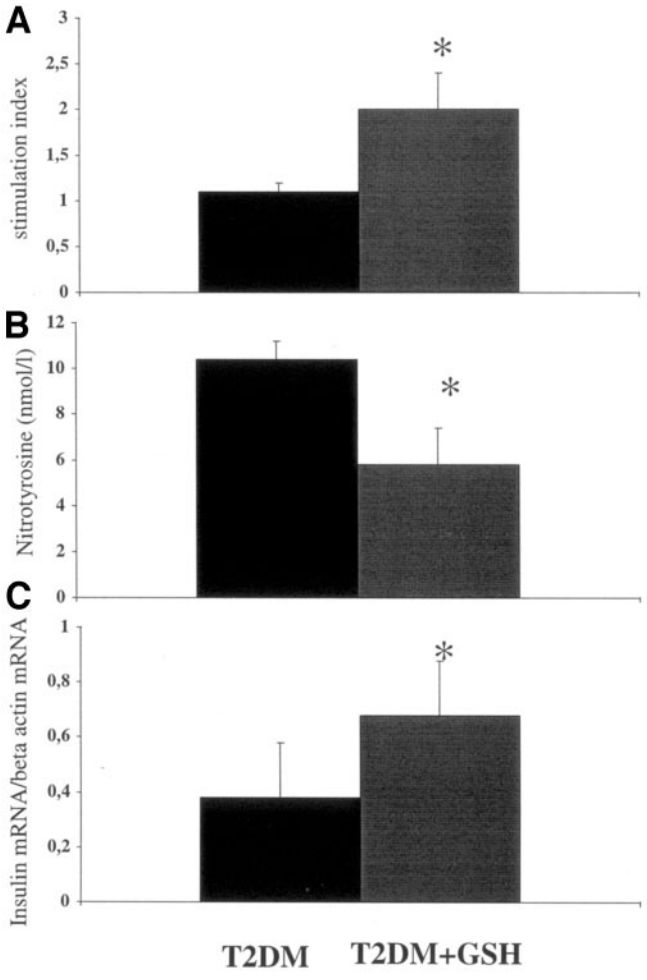

FIG. 8. Stimulation index (*A*), nitrotyrosine concentration (*B*), and insulin mRNA expression (*C*) in diabetic islets (T2DM) either not exposed or pre-exposed for 24 h to GSH. The presence of the antioxidant determined significant improvement of glucose-stimulated insulin secretion, reduction of the oxidative stress marker, and increased expression of insulin. Bars represent the means ± SD values of four separate experiments. *$P < 0.05$ vs. type 2 diabetes.

less effective in curing diabetic mice. Further work is needed to determine whether the reversibility of the defective in vitro function, as reported in our study, is accompanied by improved in vivo performance. Finally, it should be noted that in the current experiments, despite 3–4 days of culture in euglycemic medium, insulin secretion function from diabetic islets did not improve in the absence of GSH. This indicates that recovery from the possible deleterious effects of glucotoxicity may require longer times, and that the changes we observed were indeed directly caused by the pharmacological addition. It should be noted that islets prepared from the pancreata of type 1 diabetic patients show recovered function after a few days of in vitro culture (44), suggesting that the islet's native microenvironment plays a more important role in type 1 diabetes than in type 2 diabetes in negatively affecting the β-cell.

In conclusion, our results show for the first time that the functional defects of β-cells in type 2 diabetes are associated with multiple functional and molecular alterations and increased oxidative stress, which may be targets for therapeutic approaches. In fact, reduction of islet cell oxidative stress was accompanied by improved secretory capability, which suggests that the decline in β-cell function commonly observed in type 2 diabetic patients may not be relentless.

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