

Chronic Hyperglycemia Triggers Loss of Pancreatic β Cell Differentiation in an Animal Model of Diabetes*

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Differentiated pancreatic β cells are unique in their ability to secrete insulin in response to a rise in plasma glucose. We have proposed that the unique constellation of genes they express may be lost in diabetes due to the deleterious effect of chronic hyperglycemia. To test this hypothesis, Sprague-Dawley rats were submitted to a 85–95% pancreatectomy or sham pancreatectomy. One week later, the animals developed mild to severe chronic hyperglycemia that was stable for the next 3 weeks, without significant alteration of plasma nonesterified fatty acid levels. Expression of many genes important for glucose-induced insulin release decreased progressively with increasing hyperglycemia, in parallel with a reduction of several islet transcription factors involved in β cell development and differentiation. In contrast, genes barely expressed in sham islets (lactate dehydrogenase A and hexokinase I) were markedly increased, in parallel with an increase in the transcription factor c-Myc, a potent stimulator of cell growth. These abnormalities were accompanied by β cell hypertrophy. Changes in gene expression were fully developed 2 weeks after pancreatectomy. Correction of blood glucose by phlorizin for the next 2 weeks normalized islet gene expression and β cell volume without affecting plasma nonesterified fatty acid levels, strongly suggesting that hyperglycemia triggers these abnormalities. In conclusion, chronic hyperglycemia leads to β cell hypertrophy and loss of β cell differentiation that is correlated with changes in c-Myc and other key transcription factors. A similar change in β cell differentiation could contribute to the profound derangement of insulin secretion in human diabetes.

Pancreatic β cells are highly specialized cells that secrete insulin in response to a variety of stimuli, the most important being glucose (1, 2). Their correct function is dependent on the expression of a unique set of genes that allow β cells to respond to even small increases in plasma glucose levels by releasing

appropriate amounts of insulin into the circulation (3). Type 2 diabetes is characterized by the combination of insulin resistance and profound alteration in glucose-stimulated insulin secretion (4). The latter can be ascribed, at least in part, to the deleterious effect of even mild chronic hyperglycemia and elevated plasma nonesterified fatty acids (NEFA)¹ on pancreatic β cell function, a process often referred to as “gluco-lipotoxicity” (5, 6). In islets isolated from animal models of diabetes, several defects have been identified at the level of gene expression and/or enzymatic activity, but none by itself can entirely account for the β cell defect characteristic of type 2 diabetes (5, 7, 8).

Recently, the study of the transcriptional regulation of the insulin gene has led to the identification of several β cell/islet transcription factors that are important for the development of the endocrine pancreas, the tissue-specific expression of key β cell genes and maintenance of β cell differentiation (9, 10). In islets of diabetic animals and in long term culture of immortalized insulin-secreting cells, chronic exposure to high glucose can result in loss of insulin gene expression (11, 12). This loss is associated with reduced expression and/or activity of the β cell transcription factors PDX-1 and RIPE3b1 (11, 13, 14) and increased expression of the liver-adipocyte transcription factor C/EBP β (14). Similar alterations in gene expression have also been described in islets exposed *in vitro* for 2 days to high glucose and NEFA (15). We have recently hypothesized that these changes in islet gene expression could reflect a loss of differentiation of β cells chronically exposed to the diabetic milieu, leading to further deterioration of β cell function and subsequent decreased secretory function (8).

In this study, we tested the influence of chronic hyperglycemia on pancreatic β cell differentiation, using a well characterized model of chronic hyperglycemia in the rat, the 90% partial pancreatectomy (Px) (16). After an initial burst of pancreatic regeneration that lasts no longer than ~10 days, these animals have a stable population of mature islets exposed to chronic hyperglycemia from 1st week post-Px (17). Px rats are thus considered a model of β cell adaptation to increased secretory demand and exposure to the diabetic environment (5). Our results show that graded levels of chronic hyperglycemia *in vivo* leads to progressive loss of β cell differentiation that can be reversed by normalization of blood glucose levels with phlori-

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¹ The abbreviations used are: NEFA, nonesterified fatty acids; RT, reverse-transcribed; PCR, polymerase chain reaction; Px, pancreatectomy; LPx, low hyperglycemic pancreatectomy; MPx, mildly hyperglycemic Px; HPx, highly hyperglycemic Px; SPx, severely hyperglycemic Px; IAPP, islet amyloid polypeptide; GLUT, glucose transporter; HK, hexokinase; mGPDH, mitochondrial glycerol-phosphate dehydrogenase; LDH, lactate dehydrogenase; K⁺-ATP channels, ATP-sensitive K⁺ channels; VDCC, voltage-dependent Ca²⁺ channels; SERCA, sarcoplasmic reticulum Ca²⁺-ATPase; HNF, hepatocyte nuclear factor; bHLH, basic helix loop helix; ANOVA, analysis of variance; FITC, fluorescein isothiocyanate; DABCO, 1,4-diazabicyclo[2.2.2]octane.

zin, in the absence of changes in plasma NEFA. They also suggest a link between stimulation of β cell growth and their reduced state of differentiation in hyperglycemic animals.

EXPERIMENTAL PROCEDURES

Partial Pancreatectomy (Px)—Male Sprague-Dawley rats weighing 90–100 g (Taconic Farms, Germantown, NY) were anesthetized with sodium amobarbital (10% solution, 1 ml/kg,) and submitted to a partial pancreatectomy or sham surgery as described previously (16). For 90% pancreatectomy, most of the pancreatic tissue was removed by gentle abrasion with cotton applicators, leaving intact the tissue within 1–2 mm of the common bile duct and extending to the first loop of the duodenum (pancreatic remnant). In some experiments, the proportion of the gastric lobe removed was purposely varied to generate 85–95% partially pancreatectomized rats that stay normoglycemic or develop mild to severe hyperglycemia after Px. Blood was obtained weekly from the snipped tail of non-fasted rats (9–10 a.m.) in heparinized microcapillary tubes, and whole blood glucose levels were determined with a “One Touch II” glucometer (Lifescan, Milpitas, CA). Four weeks after Px, the rats were anesthetized with sodium amobarbital (10% solution, 1.1 ml/kg) and their islets isolated by collagenase digestion of the pancreatic remnants or the entire sham pancreas (11). The islets were then microdissected and handpicked under a stereomicroscope to ensure high purity of the islet preparation. The islets were maintained on ice during the entire isolation except for 20 min of collagenase digestion. All animal procedures were approved by the Animal Care Committee of the Joslin Diabetes Center.

RNA Extraction and Complementary DNA (cDNA) Synthesis—Most of the time, total RNA was extracted from islets of individual rats following manufacturer’s suggested protocols using Ultraspec (Biotex Laboratories, Houston, TX). In a few cases, however, it was necessary to pool islets from two Px rats with similar glycemia to have enough RNA for further analysis. After quantification by spectrophotometry, 500 ng of RNA was diluted to a final concentration of 0.1 μ g/ μ l and heated at 85 °C for 3 min. It was then reverse-transcribed (RT) into cDNA in a 25- μ l solution containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol, 1 mM dNTPs, 50 ng of random hexamers, and 200 units of Superscript II RNase H⁻ reverse transcriptase (Life Technologies, Inc.). Reactions were incubated for 10 min at 25 °C, 1 h at 42 °C, and 10 min at 95 °C. The final cDNA reaction products were then diluted with 50 μ l of H₂O to a concentration corresponding to 20 ng of starting RNA (20-ng RNA equivalents) per 3 μ l and stored at –80 °C (18).

Primers—Primer design was optimized for multiplex polymerase chain reaction (PCR) with Eugene™ version 2.2 (Daniben Systems, Cincinnati, OH) using the following restrictions: oligonucleotide length of 18–25 bases, GC content of 30–70%, melting temperature close to 60 °C, product length within 150–650 bases, and maximum 6–8 primer dimer formation allowed within multiplex PCR.

Semiquantitative Radioactive Multiplex PCR—Polymerization reactions were performed in a Perkin-Elmer 9600 or 9700 Thermocycler in a 50- μ l reaction volume containing 3 μ l of cDNA (20-ng RNA equivalents), 80–160 μ M cold dNTPs, 2.5 μ Ci of [α -³²P]dCTP (3000 Ci/mmol), 2.5–30 pmol of appropriate oligonucleotide primers, GeneAmp PCR buffer, and 5 units of AmpliTaq Gold DNA polymerase (Perkin-Elmer). The oligonucleotide primers and conditions used for multiplex PCR are indicated in Table I. The thermal cycle profile used was a 10-min denaturing step at 94 °C to release DNA polymerase activity (hot start PCR) followed by the number of cycles indicated in Table I and a final extension step of 10 min at 72 °C. In each set, the gene products of interest were amplified with an internal control gene (cyclophilin, α -tubulin, the rat ribosomal phosphoprotein P0 (RRPP0) or the TATA-binding protein (TBP) to correct for experimental variations between samples (RNA quantification, starting cDNA, gel loading, etc.). After removal of free [α -³²P]dCTP by gel filtration on Probequant G50 microcolumns (Amersham Pharmacia Biotech), the amplicons were separated on a 6% polyacrylamide gel in Tris borate EDTA buffer. The gel was dried, and the amount of [α -³²P]dCTP incorporated in each amplicon was measured with a PhosphorImager and quantified with the ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The amount of each specific product was then expressed relative to the internal control, giving a ratio “specific product/control gene” for each sample. These ratios were then expressed as a percent of the ratio in sham islet extracts tested in the same RT-PCR. For each sample, a negative control (RT reaction performed in the absence of RT enzyme) was performed to exclude genomic DNA contamination of the cDNA.

Validation of Semiquantitative Multiplex RT-PCR—To ensure the

validity of the measurement of mRNA levels by semiquantitative-radioactive multiplex RT-PCR, control experiments were performed using normal rat islet cDNA to show that the amount of each amplicon obtained in a multiplex PCR was independent of the presence of the other primers (cross-correlation analysis), excluding the possibility of strong interferences between primers. The number of cycles (1 min denaturation at 94 °C, 1 min at annealing temperature, and 1 min extension at 72 °C) and the final reaction conditions (MgCl₂, cold dNTP and primer concentrations, cycle number, and annealing temperature) were then adjusted to be in the exponential phase of the amplification of each product (Fig. 1A). Finally, we verified that the amount of each PCR product in a multiplex reaction increases linearly with the amount of starting cDNA (from 2.5- to 80-ng RNA equivalents), ensuring that changes in the ratio of PCR product to control gene product truly reflect a change in mRNA abundance of that gene relative to the control gene (Fig. 1B). Our results (PCR amplification and quantitation) were highly reproducible, as judged by multiple PCR determination from the same cDNA preparation (coefficient of variation <7%).

Px Rat Classification—In the first part of the study, rats sacrificed after 4 weeks were classified into 4 groups according to their averaged fed blood glucose levels 3 and 4 weeks post-Px. Low hyperglycemic rats (LPx) were below 100 mg/dl, mildly hyperglycemic rats (MPx) were within 100–150 mg/dl, highly hyperglycemic rats (HPx) were within 150–250 mg/dl, and severely hyperglycemic rats (SPx) were above 250 mg/dl (range in mM are <5.6, 5.6–8.3, 8.3–13.9 and >13.9 respectively).

In the second part of the study, Px rats were classified according to their averaged fed blood glucose levels 1 and 2 weeks post-Px in moderately hyperglycemic (<160 mg/dl) and severely hyperglycemic ($\geq$ 160 mg/dl) rats. Severely hyperglycemic Px rats were then divided in 3 groups. The first one was sacrificed 2 weeks after Px, and the other two were randomly assigned to phlorizin treatment or no treatment for the next 2 weeks.

Phlorizin Treatment—Phlorizin was dissolved in 1,2-propanediol (0.2 g/ml) and injected intraperitoneally twice a day at a dose of 0.8 g per kg body weight per day for 2 weeks (19). Sham animals received similar amounts of 1,2-propanediol as phlorizin-treated rats. To make sure that blood glucose levels were normalized throughout the day, blood sampling was always performed just before injection of the morning dose of phlorizin.

Plasma Insulin and NEFA Determination—Plasma was prepared from blood samples collected in EDTA/paraoxon (diethyl-*para*-nitrophenyl phosphate)-coated tubes, to avoid heparin stimulation of triglyceride lipase and breakdown of circulating triglycerides to glycerol and NEFA (20). Plasma insulin levels were determined by radioimmunoassay for rat insulin (Linco Research, St. Charles, MO). Plasma NEFA levels were measured by a colorimetric method (Wako Chemicals, Neuss, Germany).

Immunohistochemistry—Remnant tissue from 4-week Px (treated with or without phlorizin for the last 2 weeks) and remnant equivalent from sham Px (at least 4 rats in each group) was fixed for 2 h at room temperature in freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer and then processed for paraffin embedding. For staining of transcription factors, paraffin sections (5–7 μ m) were deparaffinized and then microwaved in citrate buffer (3 times, each for 5 min) for antigen retrieval. Sections from all blocks were then rinsed in Tris saline, incubated for 10–20 min in 0.3% Triton X-100 (Fisher) with 1% lamb serum (Life Technologies, Inc.), and following a second rinse, incubated in rabbit blocking serum (Vector, Burlingame, CA). Sections were then incubated overnight at 4 °C with Nkx6.1 antibody (gift of Pelle Serup, Hagedorn, Gentofte, DK, dilution 1:2000). After washing, sections were incubated 1 h at room temperature with donkey biotinylated anti-rabbit IgG (1:400, Jackson ImmunoResearch) as the secondary antibody followed by streptavidin-conjugated FITC (1:400, Jackson ImmunoResearch) for 1 h at room temperature. After rinsing extensively, slides were mounted with DABCO glycerol anti-fading mounting media. Images of stained sections were taken on a Zeiss LSM 410 microscope with a fluorescence filter for FITC. The sections were then washed with 0.01 M HCl to remove the attached antibodies, rinsed with distilled water, and incubated with 5% normal donkey serum (Jackson ImmunoResearch, West Grove, PA) for 30 min at room temperature. The staining procedure was repeated on the same sections with PDX-1 primary antibody overnight (Hm-253, gift of Joel Habener, Boston, dilution 1:7500) and donkey biotinylated anti-rabbit IgG (1:400, Jackson ImmunoResearch) as the secondary antibody followed by streptavidin-conjugated Texas Red (1:400, Jackson ImmunoResearch). Images of stained sections were taken with a fluorescence filter for Texas Red. Similar results were obtained with sections not previously stained for Nkx6.1.

TABLE I
Sequences of oligonucleotide primers and PCR conditions

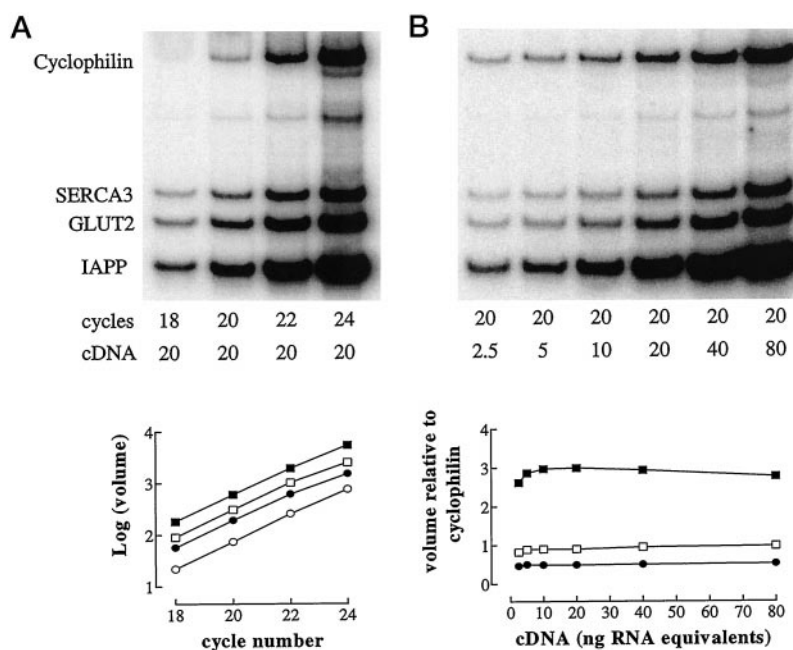
Gene name	Size	5' Oligonucleotide							3' Oligonucleotide							GenBank™	MgCl ₂	dNTP	Primer	Annealing	Cycles	Control gene	
	<i>bp</i>															<i>accession no.</i>	<i>mM</i>	<i>μM</i>	<i>nM</i>	<i>°C</i>			
Insulin (I and II)	148	TCT	TCT	ACA	CAC	CCA	TGT	CCC	GGT	GCA	GCA	CTG	ATC	CAC		J00747-8	1.5	80	200	55	15	Cyclophilin	
Somatostatin	194	ATG	CTG	TCC	TGC	CGT	CTC		TTC	TCT	GTC	TGG	TTG	GGC	TC	J00786-90	1.5	80	100-200	55	15-20	Cyclophilin	
Glucagon	245	ACC	TAG	ACT	CCC	GCC	GTG		ATG	TCT	GCG	CCC	AAG	TTC		K02808	1.5	80	200	55	15	Cyclophilin	
IAPP	154	CAA	CCG	TCA	GGT	GGA	CAA	AC	CTC	TGC	CAC	ATT	CCT	CTT	CC	M25390	1	80	200	55	20	Cyclophilin	
Amylase	198	TGG	CCT	TCT	GGA	TCT	TGC		AAA	GGT	CTG	CTT	CCT	TGG	G	M24962	1.5	80	200	55	20	Cyclophilin	
GLUT2	183	TGG	GTT	CCT	TCC	AGT	TCG		AGG	CGT	CTG	GTG	TCG	TAT	G	J03145	1	80	200	55	20	Cyclophilin	
Glucokinase, exon 1A	299	TGA	CAG	AGC	CAG	GAT	GGA	G	TCT	TCA	CGC	TCC	ACT	GCC		M25807	1.5	80	200	59	25	α-Tubulin	
Hexokinase I ^a	290	GGC	TCA	GAG	GAG	ACC	CTT	CG	CCA	GGT	CGA	ACT	TGA	ATC	AT	J03228	1.5	80	200	55	30	TBP	
mGPDH	362	AGA	TTT	CAC	ATT	TTT	GAT	GAA	AAG	TCG	TCT	CTG	GTT	GTA	CAT	A	U08027	1.5	80	400	59	25	α-Tubulin
LDH-A	249	ACA	GTT	GTT	GGG	GTT	GGT	G	CCG	GCT	CTC	TCC	CTC	TTG		X01964	1.5	80	200	59	24	α-Tubulin	
Pyruvate carboxylase	321	GCC	AAC	AGA	GGT	GAG	ATT	GCC	ATC	C	CAT	CTT	GCG	GAC	CTC	TG	U36585	1.5	80	200	55	28	α-Tubulin
Kir 6.2	199	CAT	GGA	GAA	CGG	TGT	GGG		CAG	ATA	GGA	GGT	GCG	GGC		U44897	1.5	80	200	55	26	RRPPO	
SUR1	270	ATC	ACG	GAA	GGA	GGG	GAG		TTC	CGG	CTT	GTC	GAA	CTC		L40624	1.5	80	200	55	28	α-Tubulin	
VDCCα1D	155	TTC	TCA	GGG	AAT	GGA	CGC		AGC	ACA	TCT	GTC	CAG	CCC		D38101-2	1.5	80	200	59	25	α-Tubulin	
SERCA3	222	GTA	CCC	CTA	AGC	GCC	ACC		AGC	CAC	CTC	TGG	TCT	CGG		J05086	1-1.5	80	200	55	20	Cyclophilin	
SERCA2B	185	CTC	TAC	GTG	GAA	CCT	TTG	CC	AGG	GAG	CAG	GAG	GGT	TTG		J04022	1.5	80	200	55	20	Cyclophilin	
PDX-1 (Idx-1, IPF-1, Stf-1)	169	CGG	ACA	TCT	CCC	CAT	ACG		AAA	GGG	AGA	TGA	ACG	CGG		S67435	1.5	160	200	55	20	Cyclophilin	
PDX-1 (exons 1 and 2) ^b	349	TGC	TAA	TCC	CCC	TGC	GTG	CCT	CTC	CTC	CGG	TTC	TGC	TGC	GTA	TGC	S67435	1.5	160	200	59	25	α-Tubulin
Nkx6.1 ^c	284	TCT	TCT	GGC	CTG	GGG	TGA	TG	GGC	TGC	GTG	CTT	CTT	TCT	CCA		AF004431	1.5	80	200	55	26	RRPPO
Pax6	494	GTC	TAC	CAG	CCA	ATT	CCA	CAG	CAG	CCA	TTT	CTC	TTT	CTT	TCC	T	U69644	1.5	80	200	55	26	RRPPO
Beta2/NeuroD	208	GCA	AAG	GTT	TGT	CCC	AGC		ACG	TGG	AAG	ACG	TGG	GAG		U28068	1.5	160	200	55	20	Cyclophilin	
PAN1	344	AGG	GCA	GTG	AAC	GGA	ATG		GAA	GGG	GGA	GGG	GTA	AGC		X62323	1.5	160	200	55	20	Cyclophilin	
IB1	623	CTG	TGC	CTC	TGC	CTC	CTC		TGA	CAC	CTA	TCT	TGA	CAC	CCC			1.5	160	200	59	25	α-Tubulin
HNF1α	173	ATG	AGC	CGT	CGT	CTC	CAG		GTT	GGA	TGG	CAG	CAG	GTG		M95297	1.5	80	400	59	24	α-Tubulin	
HNF4α1 and 2/5	307/337	TAC	ATC	AAC	GAC	CGG	CAG		GTG	GAG	TCT	CAG	GGG	TGG		D10554	1.5	80	200	59	24	α-Tubulin	
HNF3β	267	GGC	TCC	TTC	TGG	ACC	CTG		ACC	TCG	CTT	GTG	CTC	CTG		L09647	1.5	160	600	59	25	α-Tubulin	
SP1	265	AAG	CTC	TTC	AGG	CCC	TCC		CTG	CTG	CTA	GTC	TGC	CCC		D12768	1.5	80	200	55	25	RRPPO	
c-myc	237	AGT	TGG	ACA	GTG	GCA	GGG		ACA	GGA	TGT	AGG	CGG	TGG		Y00396	1.5	80	200	59	28	TBP	
α-Tubulin	451	CTC	GCA	TCC	ACT	TCC	CTC		ATG	CCC	TCA	CCC	ACG	TAC		J00797	1.5	80-160	50-200	55-59			
Cyclophilin	400	AAC	CCC	ACC	GTG	TTC	TTC		TGC	CTT	CTT	TCA	CCT	TCC	C	M19533	1.5	80-160	200	55-59			
RRPPO	375	TCT	CCC	CCT	TCT	CCT	TCG		CTC	CGA	CTC	TTC	CTT	TGC		X15096	1.5	80	200	55			
Mouse TBP ^c	190	ACC	CTT	CAC	CAA	TGA	CTC	CTA	ATG	ATG	ACT	GCA	GCA	AAT	CGC	D01034	1.5	80	200	55			

^a Oligonucleotide primer sequences from Ref. 7.

^b From Ref. 14.

^c TATA-binding protein from Ref. 18.

FIG. 1. Validation of multiplex PCR for amplification of IAPP (■), GLUT2 (□), and SERCA3 (●) using cyclophilin (○) as internal control. A, 50- μ l aliquots of PCR mix (containing 1 mM $MgCl_2$, 80 μ M dNTP, 200 nM of each primer, and cDNA corresponding to 20 ng of starting RNA) were run for increasing number of cycles; the products were separated on a 6% polyacrylamide gel, and band intensities were quantified using a PhosphorImager. B, the number of 20 cycles (well within the logarithmic linear range of amplification for all products) was then chosen for checking the linearity of PCR amplification with increasing amounts of cDNA (2.5- to 80-ng RNA equivalents). The relative band intensity of each product to cyclophilin remained stable within this wide range of starting cDNA. The light band between cyclophilin and SERCA3 products was observed when SERCA3 was amplified alone and is not due to interactions between different pairs of primers in the multiplex reaction.



Control serum of appropriate species gave no cross-reactions. Sections from Px and sham pancreas were stained and photographed in parallel and at the same settings. Adobe Photoshop was used to make final figures.

For lactate dehydrogenase (LDH) immunolocalization, deparaffinized non-microwaved sections were soaked in phosphate-buffered saline plus 1% lamb serum (Life Technologies, Inc.) and incubated overnight with a rabbit anti-rat liver LDH antibody (final dilution 1:250, gift of Dr. A. Völkl, Heidelberg, Germany) that recognizes the A₄- and A₃B isoforms (21). Pancreatic β cells mainly express the liver isoform 5 of LDH, a tetramer of LDH-A (LDH-A₄) (22). The sections were then incubated overnight at 4 °C, washed with Tris buffer (pH 7.4), sequentially incubated with goat anti-rabbit Ig and rabbit peroxidase anti-peroxidase conjugate (Cappel Laboratories, Cochranville, PA), stained with diaminobenzidine, and counterstained with hematoxylin. By using this procedure on liver sections, we found the expected staining pattern of LDH (21).

In another set of experiments, 4-week Px (treated or not with phlorizin) and sham islets were isolated, pelleted, and fixed for 2 h at room temperature in freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer and then processed for plastic embedding. After resin removal, the sections (1 μ m) were soaked in phosphate-buffered saline plus 1% lamb serum (Life Technologies, Inc.) and stained for the endocrine non- β cells using a mixture of antibodies as follows: rabbit anti-bovine glucagon (final dilution 1:3000, gift of Dr. M. Appel), rabbit anti-synthetic somatostatin (final dilution 1:300, made in our own laboratory), and rabbit anti-bovine pancreatic polypeptide (final dilution 1:3000, gift of Dr. R. Chance, Lilly, Indianapolis). The sections were incubated with this mixture of antibodies 40 h at 4 °C and processed as described above for LDH staining. These sections were then used to evaluate β to non- β cell ratio in isolated islets.

Electron Microscopy and Measurement of β Cell Size—Islet pellets were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer and processed for routine electron microscopy. Fifteen micrographs of each of two randomly picked islets per sample were taken at a magnification of 3800 with a Philips 301 electron microscope. β cell cross-sectional area was determined on β cells with a visible nucleus (64–120 cells/animal) by planimetry using the IPLab Spectrum image analysis software (Scanalytics, Fairfax, VA). β cell volume was estimated from these values using the formula for volume of sphere.

Data Analysis—Results are presented as means $\pm$ S.E. for the indicated number of animals or islet preparations. Statistical significance of differences between Px and sham groups was assessed by one-way ANOVA followed by a test of Dunnett for comparison with sham or by a test of Newman-Keuls for multiple comparisons. For immunohistochemistry data, representative islets from different type of animals are presented.

RESULTS

Characteristics of Px Animals—The evolution of body weight and fed blood glucose levels after Px are illustrated in Fig. 2. During the first few days after surgery, body weight gain was slightly decreased in Px rats, resulting at 1 week in a statistically significantly lower body weight in all groups of Px versus sham. Thereafter, Px animals gained weight at the same rate as sham. In contrast, changes in blood glucose were very heterogeneous among Px animals, due to deliberate, but slight, variation in the amount of pancreas removed (~85–95% Px). Three and four weeks after Px, blood glucose ranged from 68 to 317 and 72 to 336 mg/dl, respectively, whereas sham blood glucose varied from 63 to 87 and 64 to 96 mg/dl at the same time points. These values were determined on whole blood obtained in the morning from fed animals. They are lower than the corresponding plasma glucose values determined by the glucose oxidase method that range from 120 mg/dl in sham to 450–500 mg/dl in Px animals. Px rats were then classified according to their averaged blood glucose at 3 and 4 weeks post-Px as low (LPx), mildly (MPx), highly (HPx), and severely hyperglycemic (SPx) rats (see “Experimental Procedures” and Tables II and III for classification criteria). The blood glucose of SPx rats was significantly increased already by 1 week and remained significantly higher than in any other group during the entire study (Fig. 2). MPx and HPx blood glucose values were not different from sham at 1 week but increased significantly thereafter. Although these two groups could not be distinguished from each other at 2 weeks, they were significantly different 3 and 4 weeks after Px. LPx blood glucose was slightly, but not significantly, increased over sham at any time during this study.

Changes in Islet Hormone mRNA Level—The expression of 4 major islet hormones was evaluated 4 weeks after Px (Table II). As previously reported (11), insulin mRNA level was reduced by 50% in SPx islets. A similar reduction was also observed in HPx rats, but the insulin mRNA level was completely normal in MPx and LPx islets (Fig. 3). In contrast, islet amyloid polypeptide (IAPP) mRNA levels were significantly increased in LPx islets but gradually decreased from LPx to SPx which were significantly reduced by 30% compared with sham (Table II). The mRNA levels of non- β cell hormones, glucagon and

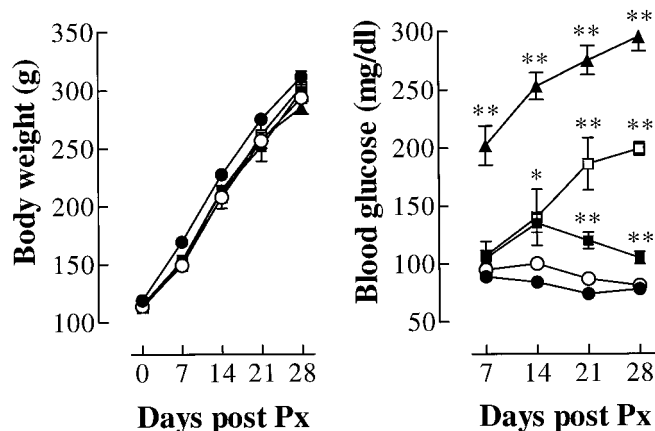


FIG. 2. **Changes in body weight and fed whole blood glucose levels after Px.** Whole blood glucose levels were determined in the fed state (9–10 a.m.) with a portable glucometer. Px rats were then classified according to their averaged blood glucose 3 and 4 weeks post-Px (see “Experimental Procedures” and Table II). Body weight gain was significantly reduced only during the 1st week after Px. Values are means $\pm$ S.E. for 11 sham ($\bullet$), 18 low hyperglycemic ($\circ$), 10 mildly hyperglycemic ($\blacksquare$), 4 highly hyperglycemic ($\square$), and 6 severely hyperglycemic ($\blacktriangle$) Px rats. Significant differences between groups were determined by one-way ANOVA followed by a test of Dunnett. * $p < 0.01$ and ** $p < 0.001$ versus controls.

somatostatin, were not significantly altered 4 weeks after Px. To verify that the decrease in insulin mRNA level was not due to variable contamination with exocrine tissue, amylase mRNA levels were measured in sham, MPx, HPx, and SPx islets and were compared with exocrine tissue obtained at the end of the islet isolation procedure. The exocrine contamination was not different between sham, MPx, HPx, and SPx (3.31 ± 0.75 ($n = 10$), 3.15 ± 0.95 ($n = 5$), 4.05 ± 0.70 ($n = 4$), and 3.59 ± 0.78 ($n = 6$) % of total islet cDNA, respectively). Because of the possibility that amylase gene expression may be altered in the diabetic state, these numbers must be regarded as only approximations. Additionally, in sections of islets isolated from H/SPx and sham immunostained for non- β cell hormones, the proportion of non- β cells to β cells appeared similar in Px and sham islets (data not shown). In our previous study, the β cell to non- β cell ratio was equal or even slightly higher in SPx than in sham islets (11). These observations exclude that an increase in exocrine contamination or decrease in β cell to non- β cell ratio artifactually accounts for the changes in β cell-specific gene expression observed in Px islet RNA.

mRNA Level of Genes Involved in Glucose Metabolism—The expression of several important determinants of β cell glucose metabolism was also evaluated 4 weeks after Px (Table II). The mRNA level of the glucose transporter 2 (GLUT2) was decreased to the same extent as insulin mRNA in HPx and SPx islets. However, it was also significantly decreased by 25% in MPx islets and moderately, although not significantly, decreased in LPx islets (Table II). Similarly, a progressive decrease with increasing blood glucose was observed for the mitochondrial glycerol-phosphate dehydrogenase (mGPDH, the rate-limiting enzyme of the glycerol-phosphate shuttle) and the anaplerotic enzyme pyruvate carboxylase (Table II, Fig. 4, sets 5 and 6). Glucokinase mRNA level was significantly reduced by 40% in SPx and HPx islets (Table II and Fig. 4, set 5) but not in MPx and LPx. Interestingly, lactate dehydrogenase-A (LDH-A) mRNA levels, which in sham islets were only about 3% that found in liver (not shown), were dramatically increased 2–3-fold in LPx and MPx islets and up to 5-fold in HPx and SPx (Table II and Fig. 4, set 3). A similar increase was also observed for hexokinase I (HK I) which was expressed at low level in sham islets (Fig. 4, set 4).

Changes in Ion Channel/Pump mRNA Level—The expression of several ion channels important for glucose-induced insulin release was also evaluated (Table II and Fig. 3, sets 1, 5, and 6). The mRNA levels of Kir6.2, the pore-forming subunit of the ATP-sensitive K^+ channels (K^+ -ATP channels) (23), were significantly reduced by 40% in SPx and HPx islets (Fig. 4, set 1) but remained essentially unaffected in LPx and MPx (Table II). In contrast, the mRNA levels of SUR1, the sulfonylurea receptor that constitutes the regulatory subunit of the K^+ -ATP channels, were not significantly altered after Px (Table II and Fig. 4, set 6). The mRNA levels of VDCC α_{1D} , the neuroendocrine isoform of the α subunit of voltage-dependent Ca^{2+} -channels, were markedly decreased with increasing glycemia after Px (Table II and Fig. 4, set 5). A similar reduction was observed for the mRNA levels of the third isoform of the sarcoendoplasmic reticulum Ca^{2+} -ATPase (SERCA3) (Table II). In contrast, the ubiquitously expressed isoform SERCA2B was only moderately reduced by 25% in SPx islets but remained totally unaffected in other Px groups (Table II).

Changes in Transcription Factor mRNA Level—We next evaluated the level of expression of transcription factors important for β cell development and differentiation. PDX-1 mRNA level was significantly reduced in HPx and SPx islets to 60% of sham (Fig. 4, set 2) and moderately reduced in LPx and MPx islets (Table II). A progressive reduction of similar, or even more severe, magnitude was also observed for the mRNA levels of Nkx6.1, Pax6, and the hepatocyte nuclear factors HNF1 α , HNF4 α , and HNF3 β (Table II and Fig. 4). In contrast, the mRNA levels of the basic helix loop helix (bHLH) factor beta2 were only decreased by 35% in HPx and SPx and remained completely normal in LPx and MPx islets. Both the islet-brain 1 transcription factor (IB1) that may play a role in the control of GLUT2 expression (24) and the bHLH factor PAN1 showed a modest decrease in all Px groups (Table II). In contrast, the ubiquitous factor SP1 remained unaffected after Px.

Reversibility of Changes in Islet Gene Expression after Px—Because correlation between blood glucose levels and changes in islet gene expression do not distinguish between cause and effect, we tested whether these changes are due to chronic hyperglycemia using phlorizin, an inhibitor of the Na^+ -glucose cotransporter in the proximal kidney tubules (25). By promoting glucosuria, this drug rapidly restored normoglycemia in severely hyperglycemic Px rats (Fig. 5). Phlorizin also slightly reduced body weight gain in Px rats but to the same extent as the vehicle did in sham (Fig. 5). As shown in Fig. 6 (left panels), the decrease in insulin, PDX-1, and Nkx6.1, and the increase in LDH-A mRNA levels were already observed in islets by 2 weeks after Px, with a similar dependence to the level of hyperglycemia as observed after 4 weeks. Correction of hyperglycemia for the next 2 weeks by phlorizin was followed by complete normalization of islet gene expression in severely hyperglycemic Px rats (Fig. 6, right panels, Fig. 7, not shown for other genes listed in Tables II–III). Administration of vehicle alone had no effect on islet gene expression or blood glucose levels in sham animals (Figs. 5–7).

Changes in Plasma NEFA and Insulin Levels after Px—The possibility that plasma NEFA or insulin levels could contribute to changes in islet gene expression after Px was also investigated. Although fed plasma insulin levels were significantly lower in severely hyperglycemic Px rats versus shams, Px plasma NEFA levels were not different from shams 2 and 4 weeks after Px (Fig. 8). After an overnight fast, plasma insulin levels were decreased significantly compared with fed values, with a large increase in plasma NEFA levels that was not different in Px and sham animals. A significant reduction in plasma insulin levels was observed in Px rats treated with

TABLE II

Changes in pancreatic islet gene mRNA levels in rats with different degree of hyperglycemia 4 weeks after Px

Px rats were classified according to their averaged 3- and 4-week blood glucose levels. The mRNA levels were compared in Px and sham by semiquantitative radioactive multiplex RT-PCR (Table I). After normalization of each product to the internal control gene level, mRNA levels were expressed in percent of sham levels observed in the same PCR. LPx, MPx, HPx, and SPx correspond to low, mildly, highly, and severely hyperglycemic Px rats. Values are means $\pm$ S.E. for the indicated number of independent determinations.

Group classification criteria	Sham, <i>n</i> = 11	LPx, <100 mg/dl, <i>n</i> = 18	MPx, 100–150 mg/dl, <i>n</i> = 10	HPx, 150–250 mg/dl, <i>n</i> = 4	SPx, >250 mg/dl, <i>n</i> = 6
Blood glucose (mg/dl) ^a	76 $\pm$ 2.4	84 $\pm$ 2.0	112 $\pm$ 2.7 ^b	192 $\pm$ 11.3 ^b	285 $\pm$ 11.4 ^b
Islet hormones					
Insulin (I + II)	100 $\pm$ 3.0	92 $\pm$ 4.1	107 $\pm$ 12.5	50 $\pm$ 7.7 ^b	51 $\pm$ 5.6 ^b
IAPP	100 $\pm$ 3.7	121 $\pm$ 4.3 ^c	112 $\pm$ 5.8	83 $\pm$ 7.1	71 $\pm$ 13.0 ^c
Glucagon	100 $\pm$ 2.1	104 $\pm$ 7.1	139 $\pm$ 28.7	157 $\pm$ 37.0	122 $\pm$ 20.6
Somatostatin	100 $\pm$ 6.8	78 $\pm$ 3.1	83 $\pm$ 12.2	82 $\pm$ 21.5	82 $\pm$ 11.9
Metabolism enzymes					
GLUT2	100 $\pm$ 2.0	88 $\pm$ 3.6	75 $\pm$ 3.7 ^b	53 $\pm$ 5.8 ^b	53 $\pm$ 5.3 ^b
GK	100 $\pm$ 0.7	83 $\pm$ 2.9	86 $\pm$ 9.8	57 $\pm$ 9.6 ^b	61 $\pm$ 6.8 ^b
HK I ^c	100 $\pm$ 2.8	189 $\pm$ 18 ^d	184 $\pm$ 23 ^d	258 $\pm$ 64.2 ^b	342 $\pm$ 80 ^b
mGPDH	100 $\pm$ 1.2	79 $\pm$ 4.1 ^c	74 $\pm$ 9.9 ^c	53 $\pm$ 5.4 ^b	55 $\pm$ 6.7 ^b
LDH-A	100 $\pm$ 8.3	249 $\pm$ 36.1 ^d	278 $\pm$ 41.4 ^d	480 $\pm$ 262 ^b	535 $\pm$ 101 ^b
Pyruvate carboxylase	100 $\pm$ 2.8	80 $\pm$ 3.9 ^c	77 $\pm$ 10.5 ^c	44 $\pm$ 7.6 ^b	40 $\pm$ 6.6 ^b
Ion channels/pumps					
Kir6.2	100 $\pm$ 1.7	91 $\pm$ 2.5	90 $\pm$ 5.1	64 $\pm$ 10.1 ^b	62 $\pm$ 6.0 ^b
SUR1	100 $\pm$ 2.2	74 $\pm$ 4.6	98 $\pm$ 15.8	86 $\pm$ 18.3	80 $\pm$ 14.4
VDCC α 1D	100 $\pm$ 2.0	78 $\pm$ 3.3 ^b	74 $\pm$ 7.8 ^b	51 $\pm$ 7.3 ^b	56 $\pm$ 6.2 ^b
SERCA3	100 $\pm$ 3.1	90 $\pm$ 2.4	80 $\pm$ 5.2 ^b	54 $\pm$ 7.3 ^b	47 $\pm$ 7.3 ^b
SERCA2B	100 $\pm$ 2.7	102 $\pm$ 3.5	97 $\pm$ 5.2	86 $\pm$ 7.2	76 $\pm$ 14 ^c

^a Averaged blood glucose values 3 and 4 weeks post-Px.^b *p* < 0.01 versus sham by test of Dunnett after one-way ANOVA.^c *p* < 0.05 versus sham by test of Dunnett after one way ANOVA.^d *p* < 0.01 versus sham when ANOVA was restricted to sham, LPx, and MPx groups.^e *n* = 3 for SPx.

TABLE III

Changes in transcription factor mRNA levels in rats with different degree of hyperglycemia 4 weeks after Px

Px rats were classified according to their averaged 3- and 4-week post-Px blood glucose levels (actual values are shown in Table II). The mRNA levels were compared between groups by semiquantitative radioactive multiplex RT-PCR (Table I). After normalization of each product to the internal control gene level, mRNA levels were expressed in percent of sham levels observed in the same PCR. LPx, MPx, HPx, and SPx correspond to low, mildly, highly, and severely hyperglycemic Px rats. Values are means $\pm$ S.E. for the indicated number of independent determinations.

Group classification criteria	Sham, <i>n</i> = 11	LPx, <100 mg/dl, <i>n</i> = 18	MPx, 100–150 mg/dl, <i>n</i> = 10	HPx, 150–250 mg/dl, <i>n</i> = 4	SPx, >250 mg/dl, <i>n</i> = 6
Blood glucose (mg/dl) ^a	76 $\pm$ 2.4	84 $\pm$ 2.0	112 $\pm$ 2.7 ^b	192 $\pm$ 11.3 ^b	285 $\pm$ 11.4 ^b
Transcription factors					
PDX-1	100 $\pm$ 2.6	80 $\pm$ 3.5 ^c	86 $\pm$ 8.8	60 $\pm$ 13.7 ^b	59 $\pm$ 6.0 ^b
Nkx6.1	100 $\pm$ 2.7	84 $\pm$ 3.6 ^c	63 $\pm$ 4.7 ^b	45 $\pm$ 6.9 ^b	40 $\pm$ 6.1 ^b
Pax6	100 $\pm$ 3.6	83 $\pm$ 4.3 ^c	67 $\pm$ 3.5 ^b	54 $\pm$ 7.0 ^b	49 $\pm$ 8.5 ^b
Beta2	100 $\pm$ 2.0	94 $\pm$ 4.1	95 $\pm$ 4.9	73 $\pm$ 13.6 ^c	65 $\pm$ 7.3 ^b
PAN1	100 $\pm$ 2.0	88 $\pm$ 2.8	85 $\pm$ 4.2 ^c	80 $\pm$ 10 ^c	74 $\pm$ 6.6 ^b
IB1	100 $\pm$ 2.8	80 $\pm$ 4.3 ^c	83 $\pm$ 8.1	74 $\pm$ 10.3 ^c	67 $\pm$ 9.3 ^b
HNF1 α	100 $\pm$ 1.7	81 $\pm$ 4.6 ^c	84 $\pm$ 7.7	56 $\pm$ 5.5 ^b	49 $\pm$ 4.9 ^b
HNF4 α 1	100 $\pm$ 2.7	77 $\pm$ 3.8 ^b	78 $\pm$ 8.3 ^c	60 $\pm$ 9.3 ^b	58 $\pm$ 4.4 ^b
HNF4 α 2/5	100 $\pm$ 2.4	70 $\pm$ 2.8 ^b	66 $\pm$ 5.8 ^b	54 $\pm$ 8.7 ^b	51 $\pm$ 4.1 ^b
HNF3 β	100 $\pm$ 3.6	72 $\pm$ 2.7 ^b	82 $\pm$ 12.8	62 $\pm$ 13.9 ^c	56 $\pm$ 6.6 ^b
SP1	100 $\pm$ 2.2	107 $\pm$ 3.8	94 $\pm$ 5.9	82 $\pm$ 2.8	105 $\pm$ 10.5

^a Averaged blood glucose values 3 and 4 weeks post-Px.^b *p* < 0.01 versus sham by test of Dunnett after one-way ANOVA.^c *p* < 0.05 versus sham by test of Dunnett after one-way ANOVA.

phlorizin but also in sham injected with vehicle alone. However, phlorizin treatment had no effect on plasma NEFA levels (Fig. 8).

Immunohistochemical Analysis of Changes in Islet Gene Expression after Px—It has been shown that changes in insulin and GLUT2 mRNA levels after Px are accompanied by parallel changes in protein levels in β cells (11). To verify that LDH-A expression is increased in pancreatic β cells, we stained sections of pancreas obtained 4 weeks after Px for LDH-A₄ (isoform 5), the main isoform expressed in islets (22). In sham pancreas, most LDH-A₄ immunoreactivity was observed in ductal epithelial cells, smooth muscle of blood vessels, lymph nodes, and in endothelial cells, even those within the islets (Fig. 9A). However, 4 weeks after Px, LDH-A₄ was significantly increased in the cytoplasm of endocrine cells throughout the islet (Fig. 9B), consistent with the increase in islet LDH-A

mRNA levels (Table II). These changes were totally reversed by 2 weeks of phlorizin treatment in Px rats (Fig. 9C). Sections of pancreas obtained 4 weeks after Px were also stained for the transcription factors PDX-1 and Nkx6.1. In control animals, PDX-1 and Nkx6.1 were strongly expressed in the nuclei of most cells in the islet core (Fig. 9, D and G). Remarkably, PDX-1 and Nkx6.1 staining was markedly decreased in islets of severely hyperglycemic Px rats (Fig. 9, E and H), and they were restored to sham levels after 2 weeks of phlorizin treatment (Fig. 9, F and I). Comparison of LDH-A₄ and Nkx6.1 staining revealed that the increase in LDH-A₄ was most pronounced in animals in which Nkx6.1 was most reduced (data not shown).

β Cell Hypertrophy and Increased *c-Myc* mRNA Levels—As can be seen in Fig. 9, Px islets were markedly hyperplastic compared with sham. Individual β cells were also hypertrophic. Four weeks after Px, their cross-sectional area, determined by

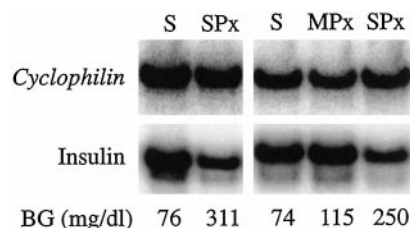


FIG. 3. Comparison of insulin mRNA levels by semiquantitative RT-PCR in islets of representative sham (S), mildly hyperglycemic (MPx), and severely hyperglycemic (SPx) rats (BG represents the averaged blood glucose values 3 and 4 weeks post-Px). Insulin mRNA was decreased by $\sim 50\%$ in SPx islets but remained unaffected in MPx islets. Cyclophilin, used as internal control gene, did not change between groups. Mean changes of insulin mRNA levels in Px versus sham islets are indicated in Table II.

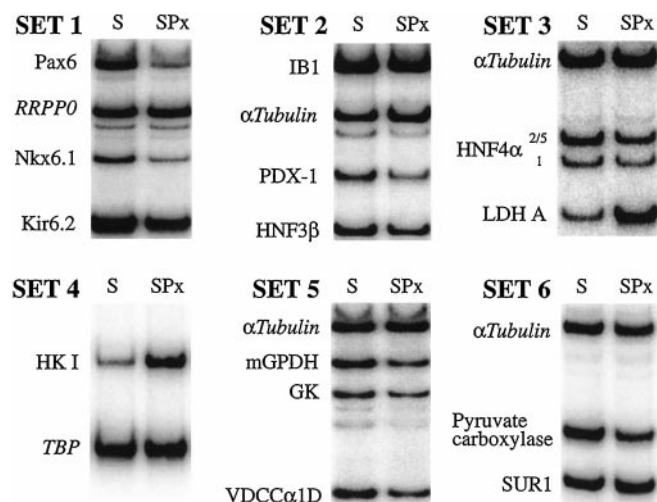


FIG. 4. Semiquantitative multiplex RT-PCR sets used to compare the mRNA levels of several transcription factors, glucose metabolism genes, and ion channels in sham and Px islets. The internal control gene used in each particular set is highlighted in *italics*. The results were obtained with islets from a representative sham (S) and a severely hyperglycemic Px rat (SPx) 4 weeks after Px (averaged 3- and 4-week blood glucose levels were 76 and 311 mg/dl, respectively).

planimetry on ultrastructural images, was increased by $\sim 50\%$ in severely hyperglycemic rats, corresponding to an 85% increase in cell volume (Table IV). In contrast, the β cell volume of phlorizin-treated Px rats was not different from shams. The proto-oncogene *c-myc* is a bHLH-leucine zipper transcription factor that stimulates cell growth and LDH-A gene expression (26). Of considerable note, *c-Myc* mRNA levels were increased in parallel to LDH-A mRNA and blood glucose levels 2 and 4 weeks after Px and returned to normal values after 2 weeks of blood glucose normalization by phlorizin treatment (Fig. 6).

DISCUSSION

Pancreatic β cells are unique in their ability to secrete insulin in response to a rise in plasma glucose, a function that, in addition to insulin, requires adequate level of expression of a unique set of genes. In the present study, we show that increasing levels of chronic hyperglycemia trigger gradual loss of β cell differentiation in a rat model of type 2 diabetes, as indicated by the following observations. First, the expression of several genes important for glucose-stimulated insulin secretion (glucose metabolism enzymes and ion channels/pumps) was gradually decreased with increasing levels of blood glucose. This reduction was already significant in the presence of low to mild hyperglycemia (<150 mg/dl), even though insulin and Kir6.2 mRNA levels were only affected in the presence of high to severe hyperglycemia (>150 mg/dl). Second, expression of

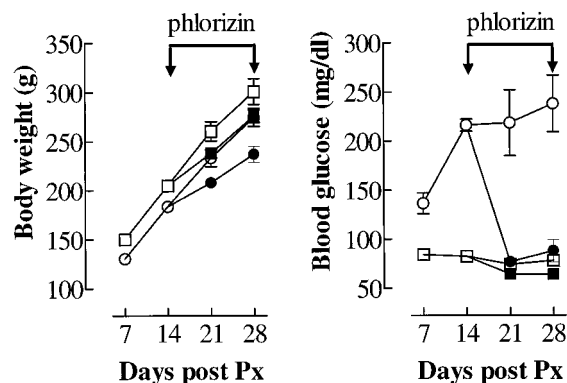


FIG. 5. Effects of phlorizin treatment on body weight and blood glucose levels after Px. Whole blood glucose levels were determined in the fed state (9–10 a.m.) with a portable glucometer. Only severely hyperglycemic Px rats were used (see “Experimental Procedures” for classification criteria). Half of Px were treated with phlorizin (0.8 g/kg body weight) for the next 2 weeks in two daily IP injections, and half of sham were injected with comparable volume of vehicle alone. Values are means $\pm$ S.E. for 6 sham ($\square$), 5 sham injected with vehicle ($\blacksquare$), 4 severely hyperglycemic ($\circ$), and 6 phlorizin-treated severely hyperglycemic ($\bullet$) Px rats.

ldh-A and *hk I*, two genes barely expressed in normal islets, was markedly increased in Px islets. Third, the levels of many, but not all, transcription factors important for β cell development and regulation of gene expression (9, 10) decreased progressively with increasing levels of hyperglycemia. That these changes in islet gene expression correspond to a loss of β cell differentiation is further supported by the observation that the mRNA levels of the proto-oncogene transcription factor *c-Myc* increase in parallel to blood glucose. An increase in *c-Myc* is likely to stimulate cell growth and inhibit cell differentiation by directly increasing the transcription of several target genes, one of which is *ldh-A* (26), or by repressing the expression of other genes (27, 28). Consistent with this hypothesis, we observed β cell hypertrophy in hyperglycemic Px rats.

Reduced Transcription Factor Expression—Expression of many β cell transcription factors decreased gradually with increasing levels of chronic hyperglycemia. HNF3 β , HNF4 α , HNF1 α , Pax6, PDX-1, and Nkx6.1 were most sensitive to chronic hyperglycemia, whereas IB1 and the bHLH factors beta2 and PAN1 were little affected, and the ubiquitous transcription factor SP1 remained totally unaffected. Although expression of none of these factors was completely abolished, their reduction could contribute with time to alteration of expression of β cell genes involved in glucose-induced insulin release, as suggested by the development of maturity-onset diabetes of the young in subjects heterozygous for mutations of *pdx-1*, *hnf4 α* , and *hnf4 α* (29–31). Thus, the expression of some of the major members of the transcriptional network responsible for β cell differentiation (9, 10) is altered in islets from chronic hyperglycemic Px rats and could contribute to the major changes observed in genes important for glucose-stimulated insulin secretion.

Changes in Gene Expression and β Cell Secretory Function—Glucose-stimulated insulin secretion is a highly regulated process in pancreatic β cells that depends on correct expression of insulin and the unique β cell metabolic and secretory machinery (2, 3). The reduction of insulin gene expression that was seen only in highly and severely hyperglycemic Px rats probably contributes to the diminished insulin secretion of these rats (32). However, the normal insulin expression in low and mildly hyperglycemic rats indicates that alteration of insulin secretion in rats with similar mild hyperglycemia (16, 33) cannot be ascribed to reduced insulin gene expression but rather to al-

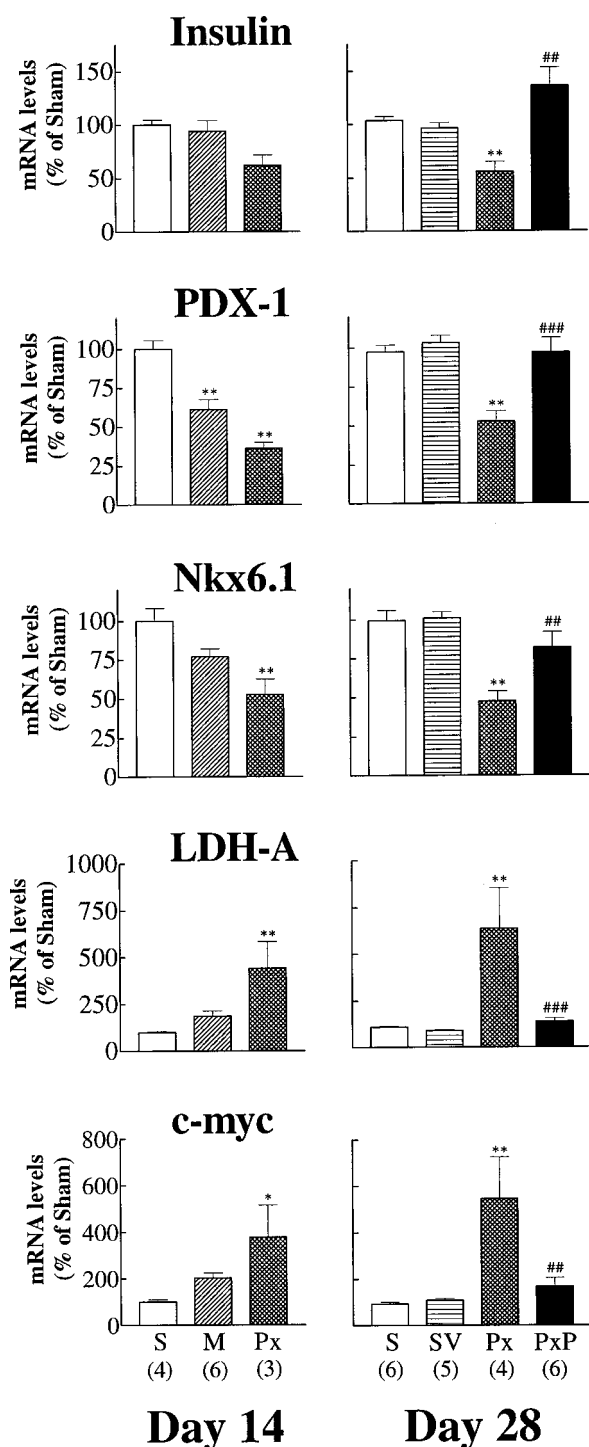


FIG. 6. Time course and reversibility of changes in islet gene expression after 90% partial pancreatectomy. After 2 weeks, Px rats were classified according to their averaged 1- and 2-week blood glucose levels in moderately hyperglycemic ($M < 160$ mg/dl) and severely hyperglycemic ($Px \geq 160$ mg/dl). Severely hyperglycemic rats were then divided in 3 groups. The first one was sacrificed 2 weeks after Px, and the other two were randomly assigned to phlorizin treatment (PxP) or no treatment (Px) for the next 2 weeks. Half of sham (S) received vehicle alone (SV). Values are means $\pm$ S.E. for the indicated number of animals. *, $p < 0.05$; **, $p < 0.01$ versus sham (sacrificed on the same day), by test of Dunnett after one-way ANOVA. ##, $p < 0.01$; ###, $p < 0.001$ versus nontreated SPx rats by test of Newman-Keuls after one-way ANOVA.

tered expression/activity of other genes important for glucose-stimulated insulin secretion. Among them, the most sensitive to chronic hyperglycemia in Px islets were GLUT2, glucoki-

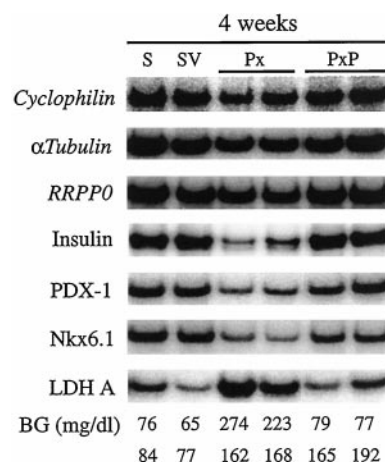


FIG. 7. Effect of 2-week normalization of blood glucose by phlorizin treatment on changes in islet gene mRNA levels after 90% Px. The different internal control genes used are highlighted in *italics*. The results were obtained 4 weeks after Px with islets from a representative sham (S), a sham injected with vehicle alone (SV), two severely hyperglycemic Px rats (Px), and two severely hyperglycemic Px rats treated with phlorizin for the last 2 weeks (PxP). BG shows the averaged blood glucose values 3 and 4 weeks post-Px on the upper line, and the averaged blood glucose values 1 and 2 weeks post-Px (before phlorizin treatment) on the bottom line.

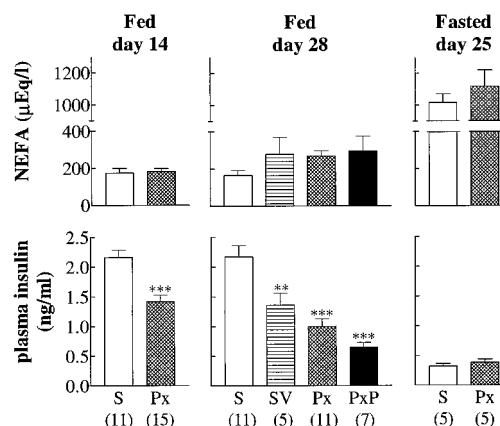


FIG. 8. Changes in plasma nonesterified fatty acid and plasma insulin levels after Px. Only severely hyperglycemic rats were used. Plasma was prepared from blood samples obtained on EDTA-paraoxon-coated tubes at 2 and 4 weeks in the fed state and after an overnight fast on day 25. Values are means $\pm$ S.E. for the indicated number of sham (S), sham-injected with vehicle (SV), severely hyperglycemic rats (Px), and severely hyperglycemic rats treated with phlorizin (PxP).

nase, mGPDH, pyruvate carboxylase, VDCC α_{1D} , and SERCA3. Their mRNA levels were all progressively reduced down to 40–60% of sham values in the presence of increasing levels of hyperglycemia, in parallel with the most sensitive transcription factors. In contrast, two genes weakly expressed in normal islets, *hk I* and *ldh-A*, increased up to 5-fold with increasing glycemia. From our results, we estimate that the ratios glucokinase/HK I and mGPDH/LDH are reduced to 25–50% of sham in LPx and MPx islets and down to 10–20% in HPx and SPx islets. These global alterations observed in Px islets could alter the preferential stimulation of oxidative metabolism by glucose and thus adversely influence glucose-stimulated insulin secretion (34–40). This conclusion relies on the premise that the changes in mRNA levels correspond to changes in protein levels and function; it is supported by parallel changes in mRNA and protein levels of LDH-A, PDX-1, and Nkx6.1. This concordance may not be verified for all genes, *e.g.* glucokinase enzymatic activity versus mRNA/protein levels (7, 41, 42).

FIG. 9. Immunohistochemistry for LDH-A₄ (immunoperoxidase, A–C), PDX-1 (Texas Red, D–F), and Nkx6.1 (FITC, G–I) on pancreatic sections from a representative 4-week sham (A, D, and G), a severely hyperglycemic Px rat (B, E, and H), and a severely hyperglycemic Px rat treated for 2 weeks with phlorizin (C, F, and I). Islets representative of islet population observed in each group of rats have been selected for illustration. The larger islets in B, E, and H are representative of hypertrophic islets observed in severely hyperglycemic animals. Magnification bars = 100 μ m.

TABLE IV

Effect of chronic hyperglycemia on β cell size 4 weeks after Px

The results were obtained with islets from two sham and four severely hyperglycemic Px rats. Two of the Px rats were injected with phlorizin for the last 2 weeks, resulting in complete normalization of their blood glucose levels. At 4 weeks, the islets were isolated, pelleted, and processed for routine electron microscopy. β cell cross-sectional area was determined by planimetry on ultrastructural images from two islets randomly selected in each pellet. Values are means $\pm$ S.E. for the number of cells (shown in parentheses) with a visible nucleus.

Class	Averaged 1–2 week blood glucose	Averaged 3–4 week blood glucose	β cell area
	mg/dl		μ m ²
Sham	87	83	87 $\pm$ 3 (70)
Sham	91	79	90 $\pm$ 3 (79)
Px	235	280	132 $\pm$ 7 (64)
Px	167	250	135 $\pm$ 7 (71)
Px + phlorizin	171	74	86 $\pm$ 3 (81)
Px + phlorizin	167	74	92 $\pm$ 3 (120)

However, the increase in HK I mRNA found in our Px rats is consistent with the increased HK activity reported in diabetic animals which probably accounts for the lower threshold for glucose-stimulated insulin secretion in Px islets and in islets overexpressing HK I (41–43). The progressive decrease of VDCC α_{1D} and SERCA3 could also contribute to the alteration of glucose-induced [Ca²⁺]_i rise and stimulation of insulin release in type 2 diabetes (7, 44, 45).

Role of Hyperglycemia in Loss of β Cell Differentiation after Px—The causal role of hyperglycemia in increased *c-myc* expression, β cell hypertrophy, and loss of β cell differentiation in the Px model is strongly suggested by their complete normalization after 2 weeks treatment with phlorizin, a drug that

normalized blood glucose without increasing plasma insulin or changing plasma NEFA levels. In fact, plasma insulin levels were even further decreased by phlorizin treatment, either because of reduced stimulation of insulin secretion by correction of hyperglycemia or possibly because of some nonspecific effect of the vehicle, 1,2-propanediol. The lack of changes in islet gene expression in shams injected with the vehicle, however, argues against a nonspecific effect of the vehicle on islets. The reversibility with phlorizin makes it also highly unlikely that the loss of β cell differentiation after Px is due to the initial burst of regeneration of islets after pancreatectomy (17). Phlorizin also corrects insulin resistance in Px rats (46). Thus, it is possible that an unknown factor changing in parallel to blood glucose and development of insulin resistance after Px is the real signal leading to alteration of β cell differentiation and secretory function, but this seems unlikely. On the other hand, plasma NEFA levels were not affected in Px rats compared with sham, indicating that they do not play any role in triggering loss of β cell differentiation after Px. However, we do not exclude the possibility that the deleterious effect of hyperglycemia on pancreatic β cell could involve alterations in intracellular lipid metabolism and accumulation of triglycerides in the cytoplasm of β cells, as seems to be the case in islets from Zucker Diabetic Fatty rats (6, 47, 48).

An inverse relationship between cell growth and their state of differentiation has previously been reported in immortalized insulin secreting cell lines (Philippe *et al.* (49) and Fleischer *et al.* (50)). Since both glucose and NEFA can induce expression of early genes in insulin-secreting cell lines (Yamashita *et al.* (51), Susini *et al.* (52), and Roche *et al.* (53)), it is tempting to speculate that plasma NEFA and hyperglycemia may be dele-

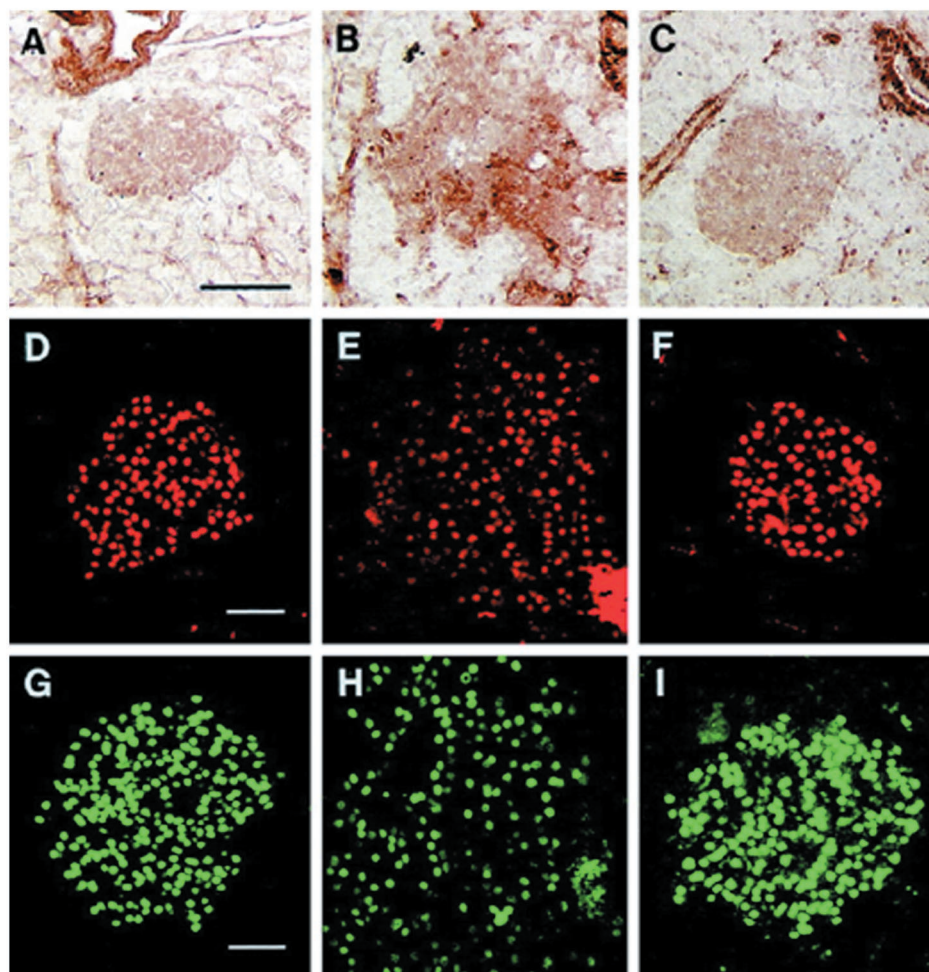


FIG. 9. Immunohistochemistry for LDH-A₄ (immunoperoxidase, A–C), PDX-1 (Texas Red, D–F), and Nkx6.1 (FITC, G–I) on pancreatic sections from a representative 4-week sham (A, D, and G), a severely hyperglycemic Px rat (B, E, and H), and a severely hyperglycemic Px rat treated for 2 weeks with phlorizin (C, F, and I). Islets representative of islet population observed in each group of rats have been selected for illustration. The larger islets in B, E, and H are representative of hypertrophic islets observed in severely hyperglycemic animals. Magnification bars = 100 μ m.

TABLE IV

Effect of chronic hyperglycemia on β cell size 4 weeks after Px

The results were obtained with islets from two sham and four severely hyperglycemic Px rats. Two of the Px rats were injected with phlorizin for the last 2 weeks, resulting in complete normalization of their blood glucose levels. At 4 weeks, the islets were isolated, pelleted, and processed for routine electron microscopy. β cell cross-sectional area was determined by planimetry on ultrastructural images from two islets randomly selected in each pellet. Values are means $\pm$ S.E. for the number of cells (shown in parentheses) with a visible nucleus.

Class	Averaged 1–2 week blood glucose	Averaged 3–4 week blood glucose	β cell area
	mg/dl		μ m ²
Sham	87	83	87 $\pm$ 3 (70)
Sham	91	79	90 $\pm$ 3 (79)
Px	235	280	132 $\pm$ 7 (64)
Px	167	250	135 $\pm$ 7 (71)
Px + phlorizin	171	74	86 $\pm$ 3 (81)
Px + phlorizin	167	74	92 $\pm$ 3 (120)

However, the increase in HK I mRNA found in our Px rats is consistent with the increased HK activity reported in diabetic animals which probably accounts for the lower threshold for glucose-stimulated insulin secretion in Px islets and in islets overexpressing HK I (41–43). The progressive decrease of VDCC α_{1D} and SERCA3 could also contribute to the alteration of glucose-induced $[Ca^{2+}]_i$ rise and stimulation of insulin release in type 2 diabetes (7, 44, 45).

Role of Hyperglycemia in Loss of β Cell Differentiation after Px—The causal role of hyperglycemia in increased *c-myc* expression, β cell hypertrophy, and loss of β cell differentiation in the Px model is strongly suggested by their complete normalization after 2 weeks treatment with phlorizin, a drug that

normalized blood glucose without increasing plasma insulin or changing plasma NEFA levels. In fact, plasma insulin levels were even further decreased by phlorizin treatment, either because of reduced stimulation of insulin secretion by correction of hyperglycemia or possibly because of some nonspecific effect of the vehicle, 1,2-propanediol. The lack of changes in islet gene expression in shams injected with the vehicle, however, argues against a nonspecific effect of the vehicle on islets. The reversibility with phlorizin makes it also highly unlikely that the loss of β cell differentiation after Px is due to the initial burst of regeneration of islets after pancreatectomy (17). Phlorizin also corrects insulin resistance in Px rats (46). Thus, it is possible that an unknown factor changing in parallel to blood glucose and development of insulin resistance after Px is the real signal leading to alteration of β cell differentiation and secretory function, but this seems unlikely. On the other hand, plasma NEFA levels were not affected in Px rats compared with sham, indicating that they do not play any role in triggering loss of β cell differentiation after Px. However, we do not exclude the possibility that the deleterious effect of hyperglycemia on pancreatic β cell could involve alterations in intracellular lipid metabolism and accumulation of triglycerides in the cytoplasm of β cells, as seems to be the case in islets from Zucker Diabetic Fatty rats (6, 47, 48).

An inverse relationship between cell growth and their state of differentiation has previously been reported in immortalized insulin secreting cell lines (Philippe *et al.* (49) and Fleischer *et al.* (50)). Since both glucose and NEFA can induce expression of early genes in insulin-secreting cell lines (Yamashita *et al.* (51), Susini *et al.* (52), and Roche *et al.* (53)), it is tempting to speculate that plasma NEFA and hyperglycemia may be dele-

terious to β cells through a common mechanism, the stimulation of cell growth and increased *c-myc* expression, along with other transcription factors (14), leading to loss of β cell differentiation. Indeed, islet hyperplasia is a common feature of many rodent models of type 2 diabetes, as are changes in expression of several genes investigated in this study (7, 11, 14, 35, 44, 45, 54, 55). Furthermore, *in vitro* experiments have shown that high glucose or elevated NEFA can have similar effects on β cell gene expression and secretory function (13, 15).

Even Px rats with only mild hyperglycemia have loss of β cell differentiation with marked alterations in islet gene expression and secretory function (33). Thus, it is possible that a similar mechanism occurs during the progression of glucose intolerance to type 2 diabetes in humans. Phlorizin treatment has been shown to correct glucose-induced insulin secretion in Px rats when secretion is expressed relative to the β cell mass remaining in the pancreas (32). Thus, complete normalization of islet secretory function occurs in parallel with correction of islet gene expression.

In conclusion, prolonged exposure to increasing levels of hyperglycemia correlates with progressive loss of β cell differentiation, as indicated by altered expression of several key islet transcription factors and other islet genes important for normal glucose-stimulated insulin secretion. Similar alterations might be responsible for at least part of the altered β cell secretory function of human diabetes. Our finding of increased *c-myc* mRNA levels provides a plausible link between stimulation of cell growth and loss of β cell differentiation in hyperglycemic animals. However, this hypothesis must be tested with genetic and more definitive approaches.

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