

Increased Expression of Antioxidant and Antiapoptotic Genes in Islets That May Contribute to β -Cell Survival During Chronic Hyperglycemia

D. Ross Laybutt,¹ Hideaki Kaneto,¹ Wendy Hasenkamp,¹ Shane Grey,² Jean-Christophe Jonas,¹ Dennis C. Sgroi,³ Adam Groff,¹ Christiane Ferran,² Susan Bonner-Weir,¹ Arun Sharma,¹ and Gordon C. Weir¹

Hypertrophy is one mechanism of pancreatic β -cell growth and is seen as an important compensatory response to insulin resistance. We hypothesized that the induction of protective genes contributes to the survival of enlarged (hypertrophied) β -cells. Here, we evaluated changes in stress gene expression that accompany β -cell hypertrophy in islets from hyperglycemic rats 4 weeks after partial pancreatectomy (Px). A variety of protective genes were upregulated, with markedly increased expression of the antioxidant genes heme oxygenase-1 and glutathione peroxidase and the antiapoptotic gene A20. Cu/Zn-superoxide dismutase (SOD) and Mn-SOD were modestly induced, and Bcl-2 was modestly reduced; however, several other stress genes (catalase, heat shock protein 70, and p53) were unaltered. The increases in mRNA levels corresponded to the degree of hyperglycemia and were reversed in Px rats by 2-week treatment with phlorizin (treatment that normalized hyperglycemia), strongly suggesting the specificity of hyperglycemia in eliciting the response. Hyperglycemia in Px rats also led to activation of nuclear factor- κ B in islets. The profound change in β -cell phenotype of hyperglycemic Px rats resulted in a reduced sensitivity to the β -cell toxin streptozotocin. Sensitivity to the toxin was restored, along with the β -cell phenotype, in islets from phlorizin-treated Px rats. Furthermore, β -cells of Px rats were not vulnerable to apoptosis when further challenged in vivo with dexamethasone, which increases insulin resistance. In conclusion, β -cell adaptation to chronic hyperglycemia and, hence, increased insulin demand is accompanied by

the induction of protective stress genes that may contribute to the survival of hypertrophied β -cells. *Diabetes* 51:413–423, 2002

Expansion of islet β -cell mass represents an important compensatory mechanism to maintain normoglycemia in the face of insulin resistance and obesity (1–5). An increase in β -cell mass accompanies insulin resistance induced in rats by glucocorticoid treatment (6) and in mice by mutation of the insulin receptor or insulin receptor substrate-1 (1). In humans, there is evidence that β -cell mass is increased in obese subjects compared with lean control subjects (7). In rodents, there is substantial evidence that increases in β -cell size (hypertrophy), replication, and neogenesis occur in situations of an increased insulin demand. For example, partial pancreatectomy (Px) in rats induces active neogenesis and β -cell replication within the first 7 days (8,9), and by 4 weeks, moderate chronic hyperglycemia produces β -cell hypertrophy (3,10). Hypertrophy has also been found in rats after chronic glucose infusion (11), in prediabetic ZDF-*fa/fa* rats with impaired glucose tolerance (2), and in pregnancy (12).

During the progression to type 2 diabetes, the capacity for compensatory β -cell expansion may be overwhelmed, leading to decompensation (2,4,5,13–15). Reduction in β -cell mass secondary to an increased rate of apoptosis has been implicated (2,13,15). Recent experiments show that female ZDF rats maintain their compensation with β -cell hyperplasia and hypertrophy, but when challenged by 48 h of dexamethasone treatment, decompensation results because of a striking increase in β -cell apoptosis (6). These results raise the question of whether hypertrophied β -cells are vulnerable to apoptosis when challenged.

It has been suggested that β -cells are particularly susceptible to apoptosis because various protective genes are expressed at low levels (16,17). We hypothesize that the induction of protective genes may be a compensatory mechanism that promotes survival of hypertrophied β -cells. We used the Px model, which undergoes compensatory growth and develops hypertrophy (3,8,10), and which serves as a model of adaptation to increased secretory demand, to examine the long-term influence of hyperglycemia on islet stress gene expression and the susceptibility of β -cells to apoptosis.

From the ¹Section of Islet Transplantation and Cell Biology, Joslin Diabetes Center, Boston, Massachusetts; the ²Immunobiology Research Center, Beth Israel Deaconess Medical Center, Boston, Massachusetts; and the ³Molecular Pathology Unit, Massachusetts General Hospital, Boston, Massachusetts.

Address correspondence and reprint requests to Gordon C. Weir, Islet Transplantation and Cell Biology, Joslin Diabetes Center, One Joslin Place, Boston, MA 02215. E-mail: Gordon.Weir@joslin.harvard.edu.

Received for publication 30 May 2001 and accepted in revised form 12 November 2001.

J.-C.J. is currently affiliated with the Unit of Endocrinology and Metabolism, University of Louvain, Brussels, Belgium.

ABC, avidin-biotin complex; DTT, dithiothreitol; HO-1, heme oxygenase-1; HPx, high hyperglycemia after pancreatectomy; HSP70, heat shock protein 70; iNOS, inducible nitric oxide synthase; LPx, low hyperglycemia after pancreatectomy; MPx, mild hyperglycemia after pancreatectomy; NF- κ B, nuclear factor κ B; 8OHdG, 8-hydroxy-2'-deoxyguanosine; PI, propidium iodide; Px, pancreatectomy; ROS, reactive oxygen species; SOD, superoxide dismutase; STZ, streptozotocin; TBP, TATA-binding protein.

TABLE 1
Sequences of oligonucleotide primers and PCR conditions

Gene Name	Size (bp)	5' Oligonucleotide	3' Oligonucleotide
HO-1	307	ATG CCC CGC TCT ACT TCC	GTG GGG TTG TCG ATC CTC
Glutathione peroxidase	301	GGT TTC CCG TGC AAT CAG	GAA CAC CGT CTG GAC CTA CC
Cu/Zn-SOD	333	AAC TGA AGG CGA GCA TGG	ATC ACA CCA CAA GCC AAGC
Mn-SOD	410	CAC CAC AGC AAG CAC CAC	TCC CAC ACA TCA ATC CCC
Catalase	173	GCT CCC AAC TAC TAC CCC AAC	CGT TTC CTC TCC TCC TCA TTC
A20	497	TTT GAG CAA TAT GCG GAA AGC	AGT TGT CCC ATT CGT CAT TCC
BCL-2	170	CCT GGC ATC TTC TCC TTC	AGA AGT CAT CCC CAG CCC
HSP70	278	ACG CAG ACC TTC ACC ACC	CGC TCG ATC TCC TCC TTG
NF- κ B (subunit p65)	144	TTT CCC CTC ATC TTT CCC TC	TGT GCT TCT CTC CCC AGG
p53	295	GCT CAC TCC AGC TAC CCG	ACC CAG CAA CTA CCA ACC C
Fas	322	TGA GCC TTG GAG ACG AAC	ATC TTG GGG GCT GTT GTG
Cyclophilin	400	AAC CCC ACC GTG TTC TTC	TGC CTT CTT TCA CCT TCC C
TBP	190	ACC CCT CAC CAA TGA CTC CTA TG	ATG ATG ACT GCA GCA AAT CGC
α -Tubulin	451	CTC GCA TCC ACT TCC CTC	ATG CCC TCA CCC ACG TAC

RESEARCH DESIGN AND METHODS

Sprague-Dawley rats (Taconic Farms, Germantown, NY) weighing ~100 g underwent 90% Px or sham-Px surgery as previously described (18). Briefly, tissue removal was performed by gentle abrasion with cotton applicators, leaving the pancreas within 1–2 mm of the common pancreatic bile duct and extending from the duct to the first part of the duodenum. Body weight and postabsorptive (morning) blood glucose levels (One Touch II glucometer, Lifescan, Milpitas, CA) were measured weekly for 4 weeks. At 4 weeks post-Px, rats were anesthetized and their islets isolated by collagenase digestion of the pancreatic remnant or sham pancreas. The islets were separated on a Histopaque density gradient and hand-picked under a stereomicroscope to ensure a pure islet preparation. Islets of similar size were used for extraction of RNA or transferred to tissue culture for further studies. Most of the time, the islet yield after Px (~50 islets) was sufficient for RNA extraction from islets of individual rats. However, in a few cases, it was necessary to pool islets from two Px rats with similar glycemia. Some experiments were performed on islet extracts from a subset of rats used in our previous study (3). In an additional set of experiments, the proportion of the pancreas removed was varied from 85 to 95% to generate rats with different degrees of hyperglycemia. Rats were classified according to their averaged 3- and 4-week blood glucose levels. In the Px rats, low hyperglycemia (LPx) was classified with blood glucose levels <100 mg/dl, mild hyperglycemia (MPx) was classified with blood glucose levels between 100 and 150 mg/dl, and high hyperglycemia (HPx) as >150 mg/dl. Plasma insulin was measured by radioimmunoassay (Linco Research, St Charles, MO) and serum 8-hydroxy-2'-deoxyguanosine (8OHdG) by enzyme-linked immunosorbent assay (Japan Institute for the Control of Age) under manufacturer-recommended conditions. Islet nitric oxide (measured as nitrite) generation was determined in culture media using Griess reagent, as previously described (19).

RNA extraction and cDNA synthesis. Total RNA was extracted from islets according to manufacturer-suggested protocols (Ultraspec, Biotecx Laboratories, Houston, TX). After quantification by spectrophotometry, 500 ng of RNA was reverse-transcribed into complementary DNA (cDNA) in a 25 μ l solution containing 5 μ l first-strand buffer (50 mmol/l Tris-HCl, 75 mmol/l KCl, and 3 mmol/l MgCl₂; Life Technologies, Grand Island, NY), 10 mmol/l dithiothreitol (DTT), 1 mmol/l dNTP, 50 ng random hexamers, and 200 units of Superscript II RNase H⁻ reverse transcriptase (Life Technologies). Reverse transcription (RT) reactions were incubated for 10 min at 25°C, 60 min at 42°C, and 10 min at 95°C. cDNA products were diluted to a concentration corresponding to 20 ng of starting RNA per 3 μ l and stored at -80°C.

Semiquantitative radioactive multiplex polymerase chain reaction. PCRs were carried out in 50- μ l volumes consisting of 3 μ l of cDNA, 1.5–2.0 mmol/l MgCl₂, 80–160 μ mol/l dNTP, 40–500 nmol of oligonucleotide primers, 2.5 μ Ci of [α -³²P]dCTP (3,000 Ci/mmol), 5 μ l GeneAmp PCR buffer, and 5 units of AmpliTaq Gold DNA polymerase (Perkin-Elmer, Foster City, CA). Table 1 shows specific concentrations of MgCl₂, dNTPs, and primers, designed with Eugene version 2.2 software (Daniben Systems, Cincinnati, OH). All reactions were performed in a 9700 Thermocycler (Perkin-Elmer), in which samples underwent a 10-min initial denaturing step to activate the DNA polymerase, followed by cycling of 1 min at 94°C, 1 min at the annealing temperature indicated in Table 1, and 1 min at 72°C. The final extension step was 10 min at 72°C. Each gene of interest was amplified along with an internal control gene (cyclophilin, TATA-binding protein [TBP], or α -tubulin). PCR products

were separated on a 6% polyacrylamide gel in Tris borate EDTA (TBE) buffer. Band intensity was measured with a PhosphorImager and quantified with ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The amount of each product was expressed relative to the internal control gene. These relative values were then used to calculate the percent of sham expression for each Px animal. PCRs were performed on RT-negative samples to exclude genomic DNA contamination for each cDNA preparation. To validate the RT-PCR analysis of each gene, control experiments were performed to adjust the PCR conditions such that the number of cycles used was in the exponential phase of amplification for all products and that each PCR product in a multiplex reaction increased linearly with the amount of starting material (from 2.5 to 80 ng RNA equivalents) (Fig. 1).

Phlorizin treatment. In one set of experiments, 2 weeks after Px, rats were randomly assigned for phlorizin treatment or no treatment for the final 2 weeks of the study period. Phlorizin was dissolved in 1,2-propanediol and injected intraperitoneally twice a day (0.8 g \cdot kg⁻¹ \cdot day⁻¹). Sham animals were either untreated or received similar amounts of 1,2-propanediol as phlorizin-treated Px rats.

Laser capture microdissection. Further gene expression analysis was performed on the β -cell-enriched central core of islets extracted from pancreas sections using the recently developed technique of laser capture microdissection. The pancreatic remnant of Px rats and the equivalent region of the pancreas of sham rats were dissected and frozen in TissueTek OCT medium (VWR Scientific Products, San Diego, CA). The tissue was sectioned at 8 μ m in a cryostat, mounted on uncoated glass slides, and frozen at -80°C. The central core of islets with diameter >100 μ m was microdissected using laser capture microdissection as previously described (20). Total RNA from microdissected samples was extracted (20), amplified by T7-based RNA amplification as described (21), and analyzed by radioactive PCR.

Immunohistochemistry. Immunohistochemical detection of heme oxygenase-1 (HO-1) protein was performed using the avidin-biotin complex (ABC) method (Vectastain ABC Kit; Vector Laboratories, Burlingame, CA). Pancreases from 4-week-old Px and sham rats were excised, fixed by immersion in 4% buffered formaldehyde, and then embedded in paraffin. Paraffin sections were incubated for 10 min with 3% H₂O₂ solution to block endogenous peroxidase and then overnight at 4°C with goat anti-HO-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:1,000 in PBS containing 1% BSA, and for 30 min with biotinylated anti-goat IgG (Vector Laboratories) diluted 1:200. These sections were then incubated with ABC reagent for 30 min, and positive reactions were visualized by incubation with the peroxidase substrate solution containing 3,3'-diaminobenzidine tetrahydrochloride.

The same sections were stained for the β -cell hormone insulin (guinea pig anti-human insulin, 1:200 dilution; Linco Research) and the α -cell hormone glucagon (rabbit anti-bovine glucagon, 1:2,000 dilution; gift of M. Appel, Worcester, MA). The secondary antibodies used for immunofluorescence were Texas Red-conjugated affinity-pure donkey anti-guinea pig IgG (1:100 dilution, Jackson ImmunoResearch) for insulin and FITC-conjugated donkey anti-rabbit IgG and streptavidin-conjugated FITC (1:100 dilution, Jackson ImmunoResearch) for glucagon.

Electrophoretic mobility shift assay. Nuclear extracts were obtained from islets (~500 islets) of 90% Px and sham rats. In brief, cells were treated with 1 ml hypotonic buffer (20 mmol/l HEPES, pH 7.9, 20 mmol/l NaF, 1 mmol/l EDTA, 1 mmol/l EGTA, 1 mmol/l Na₂P₂O₇, 1 mmol/l Na₃VO₄, and 1 mmol/l

TABLE 1
Continued

GenBank accession no.	MgCl ₂ (mmol/l)	dNTP (μmol/l)	Primer (nmol/l)	Annealing (°C)	Cycles	Control gene
J02722	1.5	160	500	62	27	Cyclophilin
X07365	1.5	80	200	59	27	TBP
M21060	1.5	80	200	56	23	α-Tubulin
Y00497	1.5	160	80	62	26	TBP
M11670	1.5	160	100	62	27	α-Tubulin
U19463	2.0	80	400	55	30	TBP
L14680	1.5	120	400	62	27	Cyclophilin
X75357	1.5	160	200	62	28	TBP
AF079314	1.5	160	400	62	26	TBP
X13058	1.5	160	150	62	27	α-Tubulin
D26112	1.5	160	400	62	30	TBP
M19533	1.5	120–160	40	62		
D01034	1.5–2.0	80–160	100–200	55–62		
J00797	1.5	160	200	56–62		

DTT); then high-salt buffer (420 mmol/l NaCl and 20% glycerol in hypotonic buffer) was added to the pellet, followed by 1 h of incubation at 4°C. The supernatants were used as nuclear extracts. Then, 1 μg nuclear extract was incubated with 2 μg poly(dI-dC) for 1 h at room temperature in 20 μl reaction buffer (10 mmol/l HEPES, pH 7.8, 0.1 mmol/l EDTA, 75 mmol/l KCl, 2.5 mmol/l MgCl₂, 1 mmol/l DTT, and 3% ficoll). The binding reaction was initiated by adding [³²P]-labeled double-stranded oligonucleotide probe. A double-stranded oligonucleotide containing nuclear factor κB (NF-κB) consensus sequence (AGT TGA GGG GAC TTT CCC AGG C) was used as a binding probe. When required, nonradioactive wild- or mutated-type competitor oligonucleotide (AGT TGA GGC GAC TTT CCC AGG C) was used. In some of the reaction mixtures, anti-NF-κB antibody (p65 and p50; Santa Cruz Biotechnology) was added to the binding reactions 1 h before the addition of the DNA probes. After the binding reactions, samples were analyzed by separation on 6% polyacrylamide gel (150 V, 1 h) in 1 × TBE buffer (45 mmol/l Tris-HCl, 45 mmol/l boric acid, and 1 mmol/l EDTA), followed by autoradiography.

Sensitivity of Px islets to β-cell toxicity in vitro. We examined the sensitivity of islets in vitro to the β-cell toxin streptozotocin (STZ) (Sigma, St. Louis, MO). Treatment of islets with 1 mmol/l STZ (dissolved in 10 mmol/l

citrate buffer) induces significant β-cell death, as previously described (22,23). Islets isolated from sham, Px, and phlorizin-treated Px rats (~50 islets) were cultured in 1640 RPMI with 1 mmol/l STZ or citrate buffer for 20 min and then for 24 h in 500 μl of fresh 1640 RPMI containing 10 mmol/l glucose supplemented with 10% FCS, 100 units/ml penicillin, and 0.1 mg/ml streptomycin sulfate. Islets were cultured in 24-well plates at 37°C with 5% CO₂. At the end of the culture period, islets were incubated for 15 min in 1 ml acridine orange (0.5 μg/ml) and propidium iodide (PI; 10 μg/ml) solution (24). Acridine orange enters cells and fluoresces green in viable cells; PI enters only dead or dying cells, binds to nucleic acids, and fluoresces red. The islets were then washed and images acquired with a Magnafire camera on an Olympus fluorescent microscope. Red and green fields were quantitated using image analysis software (IP Lab Spectrum). Results are expressed as the proportion of PI-stained area (percent PI-stained area), which represents the percentage of dead islet tissue.

Sensitivity of Px islets in vivo to dexamethasone treatment. We examined the sensitivity of Px islets in vivo to challenge with dexamethasone. At 4 weeks after sham or Px, rats received intraperitoneal injections of dexamethasone (0.4 mg/kg, once daily for 2 consecutive days) or saline. Rats were weighed, and blood glucose and plasma insulin was measured at 0, 24, and 48 h after the initial injection. Pancreases were excised, fixed in Bouin's solution, and embedded in paraffin. Sections immunostained for insulin (guinea pig anti-human insulin, 1:200 dilution; Linco Research) and treated with PI for identification of apoptotic cells by their characteristic morphology of condensed or fragmented nuclei (12).

Data Analysis. All results are presented as the means ± SE. Statistical analysis were performed using unpaired Student's *t* test or one-way ANOVA with post-test of Dunnett.

RESULTS

Changes in stress gene expression at 4 weeks after 90% Px. Blood glucose levels were significantly increased from week 1 after Px. For the period from 3 to 4 weeks post-Px, rats showed stable hyperglycemia (average blood glucose: sham 80 ± 2, *n* = 9; Px 232 ± 14 mg/dl, *n* = 7; *P* < 0.001). As previously described (3,18), weight gain was slightly decreased for the first few days post-Px, but thereafter, Px rats gained weight at the same rate as sham-operated rats (data not shown). Representative gels from RT-PCR analysis comparing stress gene mRNAs in islets 4 weeks after sham or 90% Px are shown in Fig. 2. After normalization of the specific gene to an internal control gene (cyclophilin, TBP, or α-tubulin), mRNA levels in Px islets were quantitated as a percent of sham.

Antioxidant genes. The expression of the antioxidant genes HO-1 and glutathione peroxidase was markedly increased in islets of hyperglycemic Px rats; mRNA level for HO-1 was increased ninefold (*P* < 0.05) and glutathi-

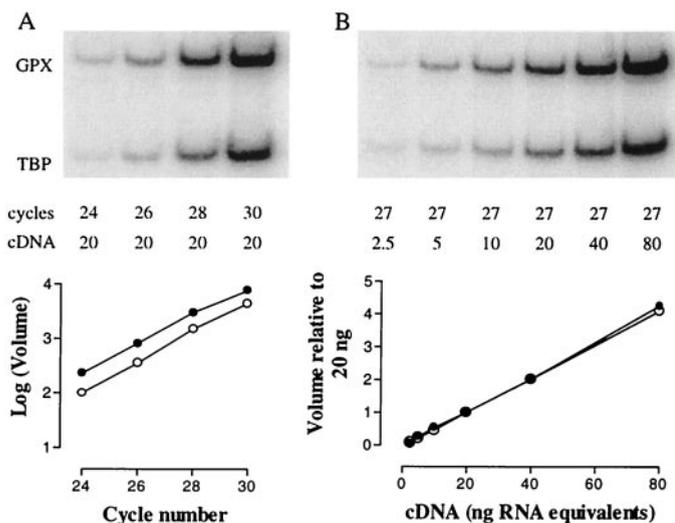


FIG. 1. Validation of multiplex PCR amplification of glutathione peroxidase (●) using TBP (○) as an internal control. **A:** cDNA corresponding to 20 ng of starting RNA was amplified by an increasing number of PCR cycles and products separated on a 6% polyacrylamide gel. Band intensity was plotted as arbitrary logarithmic units to verify that all products were within the exponential phase of amplification. **B:** Increasing amounts of cDNA (2.5–80 ng RNA equivalent) were amplified with 27 cycles of PCR to verify that each product increased linearly with the amount of starting material. GPX, glutathione peroxidase.

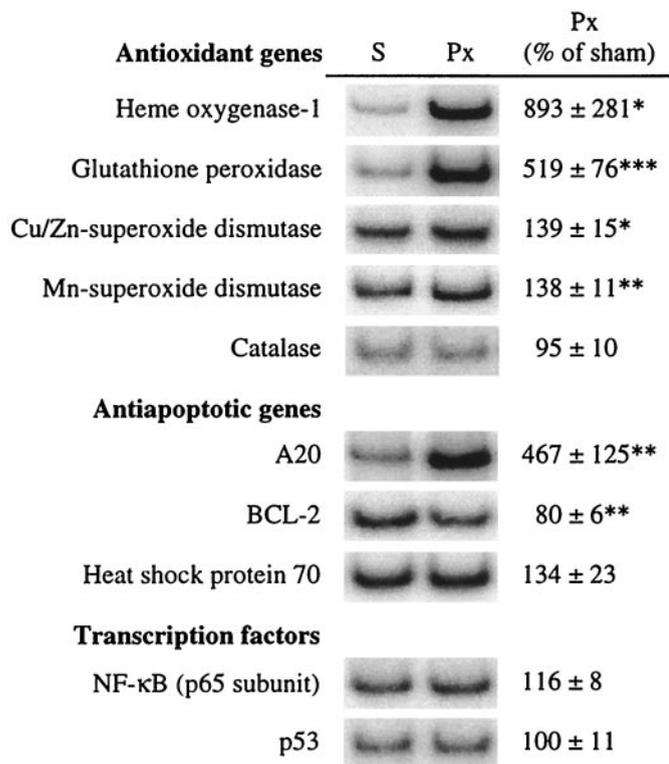


FIG. 2. Comparison of stress gene mRNA levels in islets of sham and hyperglycemic 90% pancreatectomized (Px) rats. mRNA levels were compared by semiquantitative multiplex RT-PCR. After normalization of the specific gene to an internal control gene (cyclophilin, TBP, or α -tubulin), mRNA levels in Px islets are expressed as a percent of sham. Values are means \pm SE determined from nine sham and seven Px rats. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. sham for each gene. Average blood glucose: sham 80 ± 2 mg/dl, Px 232 ± 14 mg/dl; $P < 0.001$. S, sham.

one peroxidase fivefold ($P < 0.001$) (Fig. 2). Notably, the mRNA levels of antioxidant enzymes have previously been reported to correlate with enzyme activities (17). There was also a modest but significant rise in mRNA for the antioxidant genes Cu/Zn-superoxide dismutase (SOD) (1.4-fold, $P < 0.05$) and Mn-SOD (1.4-fold, $P < 0.01$). Expression of catalase, the other antioxidant gene tested, was not altered after Px.

Antiapoptotic genes. The expression level of the antiapoptotic gene A20 was markedly increased (4.7-fold, $P < 0.01$), whereas Bcl-2 was modestly reduced (20%, $P < 0.01$) in Px islets (Fig. 2). The expression of heat shock protein 70 (HSP70) was unchanged.

Transcription factors. The mRNA level of the p65 subunit of NF- κ B did not change (Fig. 2); however, an enhanced expression of targets, such as A20 and HO-1, led us to investigate whether NF- κ B DNA binding was increased (see section on electrophoretic mobility shift assay in RESEARCH DESIGN AND METHODS) (Fig. 7). The transcription factor p53, which is implicated in mediating stress-activated apoptosis, was not increased after Px (Fig. 2).

Proapoptotic gene Fas. In contrast to the activation of potentially protective genes, expression of the proapoptosis cell surface protein Fas was modestly increased (two-fold, $P < 0.001$) after Px.

Inducible nitric oxide synthase. Using oligonucleotide primers for inducible nitric oxide synthase (iNOS) (19), no

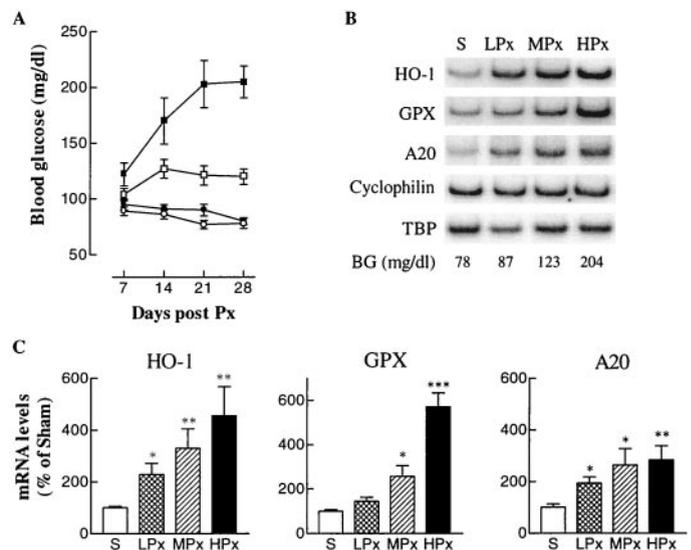


FIG. 3. Changes in stress gene mRNA levels in rats with different degrees of hyperglycemia 4 weeks after Px. **A:** Changes in blood glucose of rats in which the proportion of the pancreas removed was varied from 85 to 95% to generate rats with different degrees of hyperglycemia. Rats were classified according to their averaged blood glucose levels at 3 and 4 weeks post-Px. Values are means \pm SE for eight sham (\circ), nine LPx (\bullet), seven MPx (\square), and six HPx (\blacksquare) rats. **B:** Representative comparison of HO-1, glutathione peroxidase, and A20 mRNA levels by semiquantitative multiplex RT-PCR in islets of sham, low hyperglycemic (LPx), mildly hyperglycemic (MPx), and highly hyperglycemic (HPx) rats. Cyclophilin and TBP were used as internal control genes. The averaged blood glucose values at 3 and 4 weeks post-Px are shown at the bottom. **C:** After normalization of the specific gene to its internal control gene, mRNA levels are expressed as a percent of sham. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. sham for each gene. BG, blood glucose; S, sham.

or weak constitutive iNOS mRNA was detected in sham islets analyzed with 34 cycles of RT-PCR ($n = 9$). Under the same PCR conditions, a product for iNOS mRNA was detected in Px islets ($n = 7$). Consistent with an increased expression of iNOS, Px islets had produced more nitric oxide than sham islets after 48 h in culture (average nitrite levels in culture media: sham 3.9 ± 1.9 and Px 9.9 ± 0.7 μ mol/l per 100 islets, $n = 3$ in each group; $P < 0.05$). However, we have not determined whether iNOS mRNA in isolated islets was derived from β -cells or ductal cell contamination, as suggested in human islet preparations (25).

Note that we have verified in these samples that changes in gene expression were not caused by variations in exocrine contamination (assessed by amylase mRNA levels) between islet preparations (3). Similarly, in our previous studies, we showed that there was no change between sham and Px islets in the proportion of non- β -cells to β -cells (3,18).

Changes in stress gene expression in rats with different degrees of hyperglycemia 4 weeks after Px. By varying the proportion of the pancreas removed (85–95%), rats were generated that had blood glucose levels ranging from low (LPx, <100 mg/dl) to mild (MPx, 100–150 mg/dl) and high (HPx, >150 mg/dl) hyperglycemia (Fig. 3A). The glucose levels of rats classified as HPx were significantly ($P < 0.01$) elevated by week 1 and remained higher than any other group over the entire study period (Fig. 3A). HPx rat average blood glucose was 204 ± 16 mg/dl at 3–4 weeks post-Px. The glucose levels of rats classified as MPx

were also significantly ($P < 0.05$) increased after week 1 and remained greater than LPx and sham rats but less than HPx rats throughout the study (average blood glucose MPx 123 ± 7 mg/dl). LPx rat blood glucose levels tended to be higher than sham, though not significantly, at each time point during this study (Fig. 3A). However, the average blood glucose levels in LPx rats across the study period were significantly higher than sham. The level of hyperglycemia, even in HPx rats, was well tolerated, and there were no differences observed in weight gain among Px groups (data not shown). Notably, blood glucose levels obtained with glucose meters are ~ 30 – 40% lower than plasma levels (26).

The changes in stress gene mRNA levels in islets from sham, LPx, MPx, and HPx rats are shown in Fig. 3B and C. A progressive increase in mRNA levels for the antioxidant genes HO-1 and glutathione peroxidase and the antiapoptotic gene A20 corresponded to the degree of hyperglycemia in Px rats. The HO-1 mRNA level was significantly increased in LPx rats and was increased further ($P < 0.001$ among groups). With A20 mRNA levels, a similar association with increasing blood glucose was observed. Glutathione peroxidase mRNA levels were not significantly altered in LPx rats but were increased in MPx rats and further increased in HPx rats ($P < 0.001$).

Reversibility of changes in stress gene expression after Px. We tested whether these changes in stress gene mRNA levels were due to hyperglycemia by using phlorizin, an inhibitor of glucose reabsorption in the kidney. By blocking the Na^+ /glucose cotransporter in the proximal tubules of the kidney, phlorizin promotes glucosuria and the normalization of circulating glucose levels. As shown in Fig. 4A, hyperglycemia in Px rats was normalized for the 2-week period of phlorizin treatment. The increase in HO-1, glutathione peroxidase, and A20 mRNA levels was already observed in islets 2 weeks after Px, and it correlated with the level of hyperglycemia (Fig. 4C left panels). The normalization of blood glucose in Px rats for the next 2 weeks completely reversed the changes in expression of A20 and glutathione peroxidase and reversed HO-1 mRNA to a level that was not significantly different from sham (Fig. 4B and C, right panels). Similarly, Fas and iNOS mRNA levels were increased at 2 weeks after Px and reduced after phlorizin treatment (data not shown). The phlorizin vehicle had no effect on blood glucose levels or islet gene expression in sham-treated rats (Fig. 4).

Changes in gene expression in tissue extracted from the islet core using laser capture microdissection. Changes that were parallel to those in whole islets were observed in β -cell-enriched tissue extracted by targeting the central core of islets in sectioned frozen pancreas using laser capture microdissection. A clear increase was observed in HO-1, glutathione peroxidase, A20, iNOS, and Fas mRNA in the islet core tissue extracted from Px rats ($n = 3$) compared with no or weak constitutive detection in the islet core of sham pancreas ($n = 3$) (Fig. 5).

Immunohistochemistry. To verify that the changes in mRNA levels were accompanied by parallel changes in protein levels in β -cells, sections of pancreas obtained 4 weeks after Px were stained for HO-1; increased staining was observed in the cytoplasm of endocrine cells through-

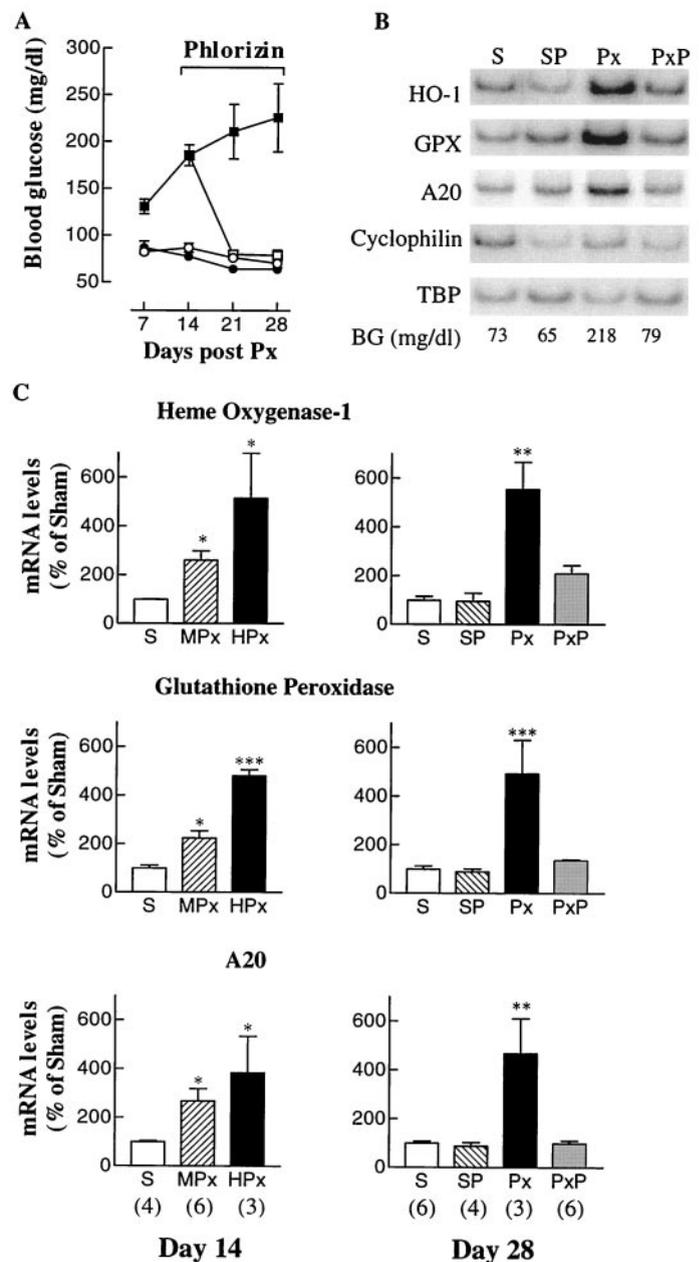


FIG. 4. Time course and reversibility of changes in islet gene expression after 90% Px. At 2 weeks post-Px, rats were either killed or randomly assigned to an untreated or a phlorizin-treated ($0.8 \text{ g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$) group for the final 2 weeks. As control, sham animals received a similar volume of vehicle (1,2-propanediol). **A:** Effect of phlorizin treatment on blood glucose levels after Px. Values are means \pm SE for three sham (\circ), four sham vehicle-treated (\bullet), three hyperglycemic untreated Px (\blacksquare), and six phlorizin-treated Px (\square) rats. **B:** Representative comparison of HO-1, glutathione peroxidase, and A20 mRNA levels by semiquantitative multiplex RT-PCR in islets of sham, sham vehicle-treated, hyperglycemic untreated Px (Px), and phlorizin-treated Px rats. Cyclophilin and TBP were used as internal control genes. Averaged blood glucose values (mg/dl) at 3 and 4 weeks post-Px are shown at bottom. **C:** Changes in mRNA levels at two (left panels) and 4 weeks (right panels) after Px. After normalization of the specific gene to its internal control gene, mRNA levels are expressed as a percent of sham. Data are means \pm SE for the indicated number of experiments. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs. sham for each gene. BG, blood glucose; PxP, phlorizin-treated Px rats; S, sham; SP, sham vehicle-treated.

out the islets from Px rats (Fig. 6B), revealing a clear increase of intensity compared with the controls (Fig. 6A). Staining for HO-1 was increased in areas of the islet that

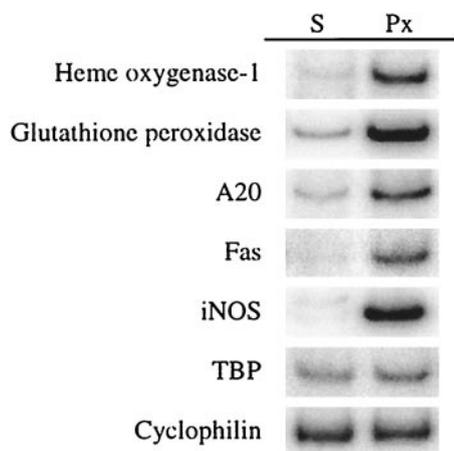


FIG. 5. Comparison by RT-PCR of stress gene mRNA levels in the β -cell-enriched central core of islets. The islet core tissue was extracted from pancreas sections of sham and hyperglycemic Px rats using laser capture microdissection. S, sham.

also stained for insulin (Fig. 6D), indicating that HO-1 expression is increased in the β -cells of Px islets; however, this was not clear for the α -cells.

Electrophoretic mobility shift assay. To assess whether diabetes induced by Px was altering NF- κ B DNA binding, we performed electrophoretic mobility shift assays using islet nuclear extracts. In the islets of sham and Px rats with low-level hyperglycemia (LPx), NF- κ B DNA binding was not detected, but in Px rats with high hyperglycemia (HPx), a marked increase of NF- κ B DNA binding was observed. As shown in Fig. 7, the band was eliminated by the wild-type competitor but not by mutated-type competitor. It was also eliminated by two kinds of NF- κ B antibody (p65 and p50), thus indicating that the band includes NF- κ B protein.

Increased oxidative stress marker (8-OHdG) in serum of Px rats. At 4 weeks after surgery, 8-OHdG levels in serum were increased in highly HPx rats but not in LPx rats compared with sham rats (Fig. 8).

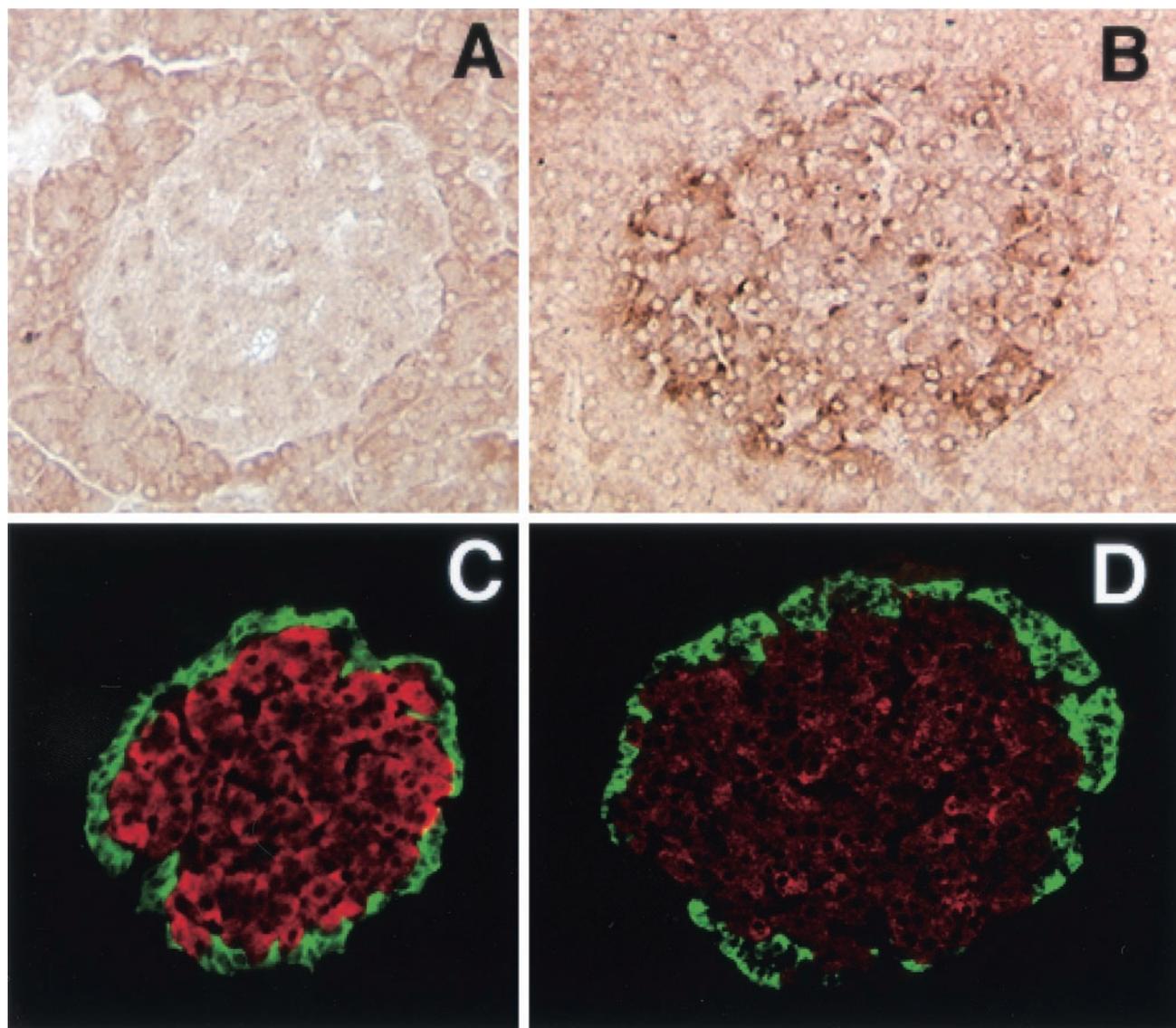


FIG. 6. Staining for HO-1 (A and B) and for insulin (red) and glucagon (green) (C and D) on pancreatic sections from a representative 4-week sham (A and C, same islet) and hyperglycemic Px rat (B and D, same islet).

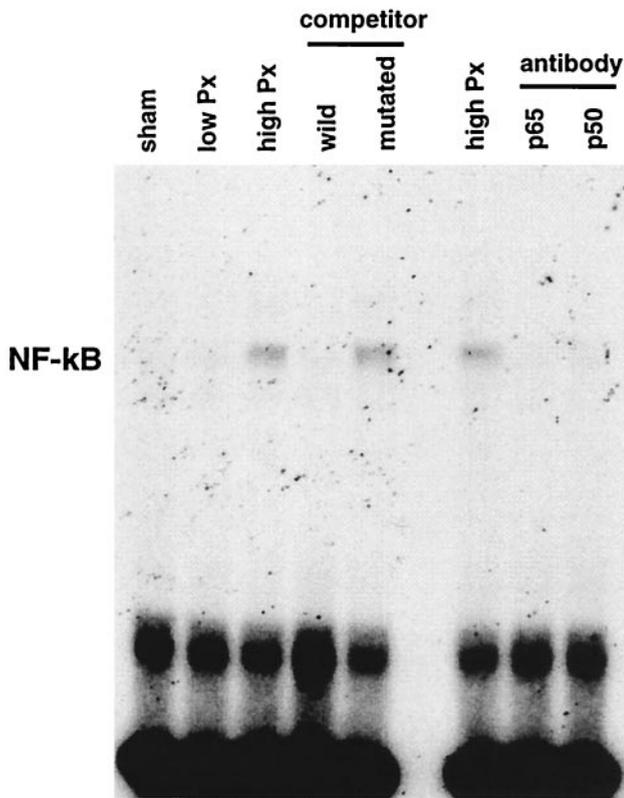


FIG. 7. Increased NF- κ B DNA binding activity in islets of hyperglycemic Px rats. At 4 weeks after surgery (90% Px and sham), nuclear extracts were obtained from the islets, and gel-shift assays were performed. A double-stranded oligonucleotide containing NF- κ B consensus sequence was used as a binding probe (AGT TGA GGG GAC TTT CCC AGG C). In some of the binding assays, nonradioactive wild- or mutated-type competitor oligonucleotide (AGT TGA GGC GAC TTT CCC AGG C) or anti-NF- κ B antibody (p65 or p50) were added. Similar results were obtained in two independent experiments.

Reduced sensitivity of Px islets in vitro to β -cell toxicity. We evaluated the sensitivity of isolated islets to a β -cell toxin, STZ. Without STZ exposure, the proportion of dead islet was similar in sham and Px islets after 24 h in culture (Fig. 9A). Islets from HPx rats showed reduced sensitivity to the toxic effect of 1 mmol/l STZ; percent PI-stained area in STZ-treated Px islets was significantly lower than in STZ-treated sham islets (Fig. 9B). Sensitivity to STZ toxicity was restored in phlorizin-treated Px islets;

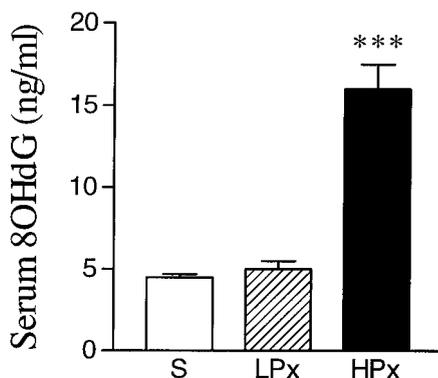


FIG. 8. Increased 8-OHdG level in serum of hyperglycemic Px rats. At 4 weeks after surgery (90% Px and sham), 8-OHdG levels in serum were measured using an ELISA Kit. Data are expressed as means \pm SE ($n = 4$). *** $P < 0.001$ vs. sham and LPx rats.

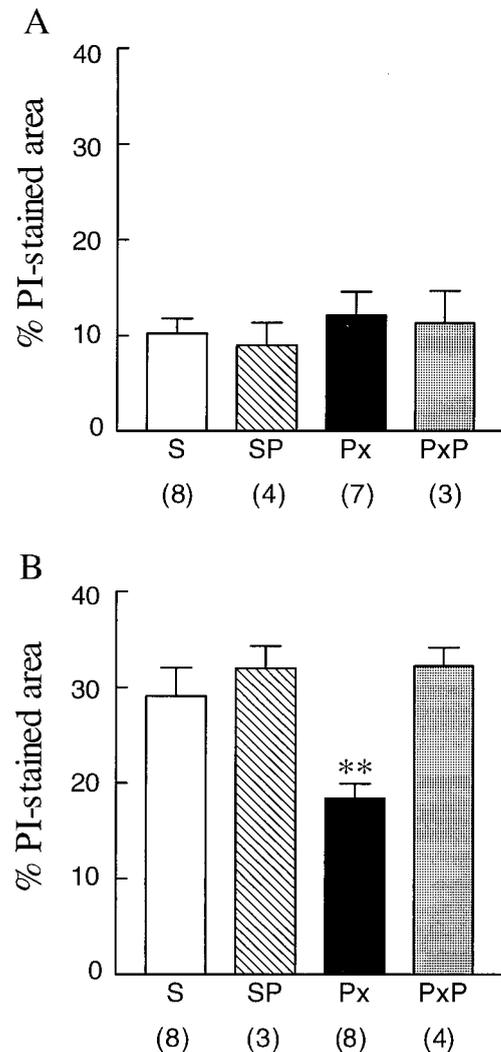


FIG. 9. Reduced sensitivity of Px islets to STZ-induced toxicity. Islets isolated from sham, Px, and phlorizin-treated Px rats were cultured in the absence (A) or presence (B) of 1 mmol/l STZ for 20 min and then without STZ for 24 h. Viability was assessed with acridine orange and PI and quantitated as the proportion of dead islet tissue (percent PI-stained area) using IP Lab Spectrum image analysis. Data are expressed as means \pm SE for the indicated number of experiments. ** $P < 0.01$ vs. STZ-treated sham. S, sham; SP, sham vehicle-treated.

percent PI-stained area in phlorizin-treated Px islets after STZ exposure was not significantly different from STZ-treated sham islets (Fig. 9B). In other experiments, at 24 h after exposure to a higher dose of STZ (10 mmol/l), islets from both Px and sham rats were completely destroyed (data not shown).

Sensitivity of Px islets in vivo to dexamethasone treatment. We next evaluated the sensitivity of Px islets in vivo to challenge with dexamethasone. The body weight of dexamethasone-treated sham and Px rats fell slightly (Table 2). Blood glucose levels of dexamethasone-treated sham rats did not change compared with saline-treated shams, but the plasma insulin concentration increased (Table 2). Blood glucose levels increased dramatically after dexamethasone treatment of Px rats; however, plasma insulin levels did not change (Table 2). Therefore, Px rats did not augment insulin secretion to further compensate for dexamethasone-induced insulin resistance. However, we were surprised to find there was no

TABLE 2

Effect of in vivo dexamethasone treatment on body weight, blood glucose, and plasma insulin at 4 weeks after sham or Px

	Sham saline	Sham-Dex	Px-saline	Px-Dex
<i>n</i>	3	3	3	3
Body weight (g)	316 ± 18	293 ± 20	308 ± 7	282 ± 13
Blood glucose (mg/dl)	103 ± 4	109 ± 2	168 ± 22*	327 ± 33**†
Plasma insulin (ng/ml)	1.3 ± 0.1	2.3 ± 0.3*	1.6 ± 0.5	1.3 ± 0.5

Data are means ± SE. Body weight, blood glucose, and plasma insulin were measured 48 h after initial administration of dexamethasone (0.4 mg/kg, once daily for 2 consecutive days) or saline. * $P < 0.05$, † $P < 0.01$ vs. saline-treated sham rats; ‡ $P < 0.05$ vs. saline-treated Px rats. Dex, dexamethasone.

observable change in the frequency of apoptotic β -cells in pancreata from dexamethasone-treated Px rats; apoptotic nuclei were barely detectable in islets within the pancreata of three dexamethasone-treated Px rats, similar to dexamethasone-treated sham and control saline-treated sham and Px rats (the low frequency of apoptotic β -cells precluded quantification in all groups). Therefore, Px rats, as in female ZDF rats (6), failed to compensate for dexamethasone-induced insulin resistance; however, in contrast to female ZDF rats, there was no increase in β -cell apoptosis.

DISCUSSION

Our results show that graded levels of chronic hyperglycemia in vivo leads to progressively increased expression of several stress genes that can be reversed by normalization of blood glucose levels with phlorizin. This suggests that induction of stress gene expression is part of the adaptation of β -cells to chronic hyperglycemia. This phenotype was associated with reduced sensitivity to the β -cell toxic agent STZ, raising the possibility that at least some of the activated genes have protective effects, which may be important for the survival of these hypertrophied β -cells.

Loss of β -cell differentiation in Px rats. We have previously shown that chronic hyperglycemia triggers a decrease in the expression of genes important for glucose-stimulated insulin secretion, β -cell development, and the regulation of β -cell gene expression (3). Concomitantly, several metabolic genes that are normally expressed at low levels, such as lactate dehydrogenase A (LDH-A) and hexokinase, are markedly increased and could, in theory, interfere with normal β -cell function. Indeed, islets from Px rats display a specific defect in glucose-induced insulin secretion, whereas secretion in response to other secretagogues and meals is better maintained (18,27). Thus, plasma insulin levels in Px rats are similar to controls in absolute terms, though inadequate with respect to glycemia (Table 2). The present study shows that expression of genes involved in antioxidant and antiapoptotic defense pathways may be an integral part of this dedifferentiation process. Several stress genes were induced, whereas others were not. Although it is unclear as to why the stress genes were differentially regulated in islets under hyperglycemic conditions, those that were induced may promote β -cell survival during times of stress. Some studies show that increasing glucose levels can promote the survival of β -cells (28), whereas others suggest that high glucose can induce β -cell apoptosis (29). From the present study in Px rats, we propose that under conditions of chronic hyperglycemia, β -cells can develop a phenotypic alteration that, in effect, trades function for survival.

Regulation of stress gene expression in islets. Stress gene mRNA levels in β -cells are considerably lower than in the liver and other tissues (16,17). Exposure of islets to high glucose (30 mmol/l) for 48 h in culture does not alter glutathione peroxidase, Cu/Zn-SOD, Mn-SOD, or catalase expression (17), but some indications of an increase in heme-oxygenase-1 expression are observed (17; J.-C.J, unpublished observations). Our data suggest that the effect of high glucose on stress gene mRNA levels is manifest in the long term, eventually reproducing an effect similar to that in vascular endothelial cells (30) and cardiac tissue (31). The five- to ninefold upregulation of protective genes may improve the cell defense status so that they are comparable to levels found in other tissues. Interestingly, incubation of rat islets in high glucose for a short period (60 min) was reported to induce SOD and glutathione peroxidase activity (32). We observed no change in mRNA levels of catalase in islets after Px, in contrast with findings in cardiac tissue of diabetic rats (31), suggesting that antioxidant enzymes may be regulated in a tissue-specific manner. There is also a species difference in the level of islet stress gene expression (16) that may have a critical influence on the lower susceptibility of normal human islets to cytotoxic damage compared with rat islets (33,34). Although on one hand, exposure of islets to combinations of cytokines causes β -cell death (35,36), culture of dispersed rat β -cells with the cytokine interleukin-1 β was shown to stimulate the expression of HO-1, Mn-SOD, A20, and HSP70, which was associated with reduced sensitivity to the toxic effects of alloxan and STZ (23).

Chronic hyperglycemia causes oxidative stress. The induction of stress gene expression in response to high glucose may be mediated by increased production of reactive oxygen species (ROS). ROS can be produced as a consequence of increased protein glycation, which can occur with chronic hyperglycemia (37–39); we found an increased level of 8OHdG in serum of HPx rats but not in LPx rats (Fig. 8). Moreover, there is evidence to suggest that hyperglycemia causes oxidative damage in β -cells of the Goto-Kakizaki (GK) rat model of nonobese type 2 diabetes (40), and increased levels of 8OHdG have been reported in the blood of people with type 2 diabetes (41,42). Furthermore, treatment of diabetic rats with antioxidants provides some protection from glucose toxicity (43–45). ROS can cause damage to cells by oxidizing nucleic acids, proteins, and membrane lipids. However, they may activate genes involved in cell defense as well as cell damage (46–48). HO-1, the inducible isoform of heme oxygenase, is an enzyme that catalyzes the NADPH-dependent decomposition of heme to form the antioxidant

biliverdin, iron, and carbon monoxide (CO) (49–51). These products may be important for protecting cells because bilirubin formed from biliverdin can inhibit the oxidation of unsaturated fatty acids and scavenge ROS; iron can lead to the formation of ferritin, which provides an iron detoxification mechanism; and CO can suppress production of cytokines and may play a role in the regulation of insulin release.

The generation of ROS may be important for the activation of the redox-sensitive transcription factor NF- κ B (46,47). Upon activation, NF- κ B may be important in promoting the transcription of stress genes that could be pro- or anti-apoptotic (19,52). For example, NF- κ B binding sites are found in the promoter regions of the HO-1 (53) and A20 genes (54). The inhibitory action of A20 on apoptosis may be due to feedback regulation of NF- κ B (19,55). The expression of A20 in islets, as in other cell types (endothelial cells, fibroblasts, and B-cells), confers resistance to cytokine-induced apoptosis (19,55). Not addressed in this study is the apparent paradox of increased expression of A20 and activation of NF- κ B. It is unclear how an adaptation in this chronic situation allows NF- κ B activation and increased A20, but perhaps the level of induction of A20 is not sufficient to fully repress NF- κ B.

The generation of nitric oxide may provide an important mechanism for the impaired glucose-induced insulin secretion and altered gene expression in Px rats. Studies by Ma et al. (56) have shown that nitric oxide can impair islet glucose utilization and attenuate glucokinase expression. **Reduced sensitivity of Px islets to STZ-induced toxicity.** Several mechanisms may contribute to the clear differential sensitivity of Px and sham islets to STZ. Conceivably, reduced GLUT2 expression in Px islets could decrease the uptake of STZ (57). Arguing against this, the likelihood is that GLUT2 is not reduced to a level sufficient to affect glucose uptake (58); therefore, presumably, the uptake of STZ will be similarly unaffected. Another possibility is that the altered phenotype of Px islets may reduce STZ-induced depletion of NAD⁺, analogous to heat shock (22). Alternatively, the increased expression of antioxidants may confer greater scavenging capacity of ROS. Increased expression of glutathione peroxidase, SOD, and catalase accompanies STZ resistance in an inbred mouse strain (ALR/Lt), suggesting that antioxidant status can control susceptibility to β -cell toxicity (59). The possible ways in which STZ can produce toxicity are by alkylation of DNA, proteins, and membrane lipids; by blocking removal of O-GlcNAc from intracellular proteins (60); or by serving as a nitric oxide donor, inducing DNA strand breaks (61,62).

Differential sensitivity of islets from Px and ZDF rats to apoptosis. Studies in ZDF rats show that the impaired glucose-tolerant females with compensated hypertrophied β -cells become frankly diabetic after dexamethasone treatment, in part because of an increase in β -cell apoptosis (6). In contrast, we found that the hypertrophied β -cells at the 4-week time point after Px were not susceptible to apoptosis after the same dexamethasone treatment. The ZDF model has the confounding influence of a specific genetic mutation and probably an intrinsic defect in their β -cells that may be responsible for their susceptibility to apoptosis (2,63,64). β -Cell apoptosis in ZDF rats was

linked to a marked reduction in Bcl-2 expression, increased plasma fatty acids, increased islet triglycerides, and ceramide accumulation (13,14). In contrast, in Px rats Bcl-2 mRNA in islets was only modestly reduced (Fig. 2) and plasma fatty acid levels unaltered (3). To our knowledge, expression of other antiapoptotic and antioxidant genes has not been studied in ZDF islets. Another potentially important consideration is that β -cells of ZDF rats are replicating at an increased rate (2,6), which may increase their susceptibility to apoptosis, whereas the β -cell replication rate at the 4-week time point after Px is similar to controls (10).

In conclusion, chronic exposure of islets to increasing levels of hyperglycemia correlates with increasing mRNA levels of several stress genes. The changes in stress gene expression were reversed by normalizing glycemia with phlorizin, strongly suggesting the specificity of hyperglycemia in eliciting the response. The changes in protective gene expression correlate with the presence of β -cell hypertrophy (3,10), raising the possibility that they contribute to the survival of those β -cells that have adapted to a hyperglycemic environment.

ACKNOWLEDGMENTS

The Diabetes Endocrinology Research Center Core facilities were supported by Grant DK-36836 from the National Institutes of Health. This work was supported by National Institutes of Health Grant DK-35449. D.R.L. is a recipient of a post-doctoral fellowship from the Juvenile Diabetes Foundation. H.K. is a recipient of a fellowship from the Japan Society for the Promotion of Science for Japanese Junior Scientists. A.S. is a recipient of a Career Development Award from the American Diabetes Association.

We thank Judy Lebet for expert technical help, the Joslin RIA Core for insulin assay, and the Animal Care Core.

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