The pancreas is a heterogeneous organ mixed with both exocrine and endocrine cells. The pancreas is involved in metabolic activities with the endocrine cells participating in the regulation of blood glucose, while the exocrine portion provides a compatible environment for the pancreatic islets and is responsible for secretion of digestive enzymes. The purpose of this study was to identify pancreatic proteins that are differentially expressed in normal mice and those with diet-induced type 2 diabetes (T2DM). In this study, C57BL/6J male mice fed a high fat diet became obese and developed T2DM. The pancreatic protein profiles were compared between control and diabetic mice using two-dimensional gel electrophoresis. Differentially expressed protein “spots” were identified by mass spectrometry. REG1 and REG2 proteins, which may be involved in the proliferation of pancreatic beta cells, were up-regulated very early in the progression of obese mice to T2DM. Glutathione peroxidase, which functions in the clearance of reactive oxidative species, was found to be down-regulated in the diabetic mice at later stages. The RNA levels encoding REG2 and glutathione peroxidase were compared by Northern blot analysis and were consistent to the changes in protein levels between diabetic and control mice. The up-regulation of REG1 and REG2 suggests the effort of the pancreas in trying to ameliorate the hyperglycemic condition by stimulating the proliferation of pancreatic beta cells and enhancing the subsequent insulin secretion. The down-regulation of glutathione peroxidase in pancreas could contribute to the progressive deterioration of beta cell function due to the hyperglycemia-induced oxidative stress. Molecular & Cellular Proteomics 4:1311–1318, 2005.

The pathological changes involved in T2DM with respect to pancreatic islets include hormone secretion dysfunction, proliferation of islet cells during the early phase, and exhaustion of beta cells. Many studies have shown that the exocrine portion of the pancreas secretes proteins that are important in the function and regeneration of beta cells (1–4).

Type 2 diabetes mellitus (T2DM) is a disease characterized by the inability of the body to properly regulate blood glucose, leading to high blood glucose levels and also disturbed metabolism of other substances such as fat and protein. Obesity and T2DM are closely linked (5). Obesity is caused by a combination of diet, sedentary lifestyle, and genetic factors. In most cases, obesity occurs before the onset of T2DM. Although obesity does not result in diabetes in normal individuals with compensated insulin secretion, it can cause insulin resistance, hyperinsulinemia, and subsequent diabetes in susceptible subjects with an unidentified genetic predisposition. The purpose of this study was to detect any differential pancreatic proteins that are associated with obesity/diabetes in a mouse model of diet-induced diabetes.

Several animal models of diabetes have been used to study obesity-induced diabetes. The commonly used genetic models, such as ob/ob (obese) and db/db (diabetic) mice, have mutations in the leptin structural gene (ob) and in the leptin receptor gene (db), respectively (6–8). The Zucker diabetic fatty rats (fa/fa) also possess mutations in the leptin receptor gene (9). However, none of these models of diabetes with known genetic defects reflects the disease in humans because these gene mutations are rare in the general population. Experimental animal models of T2DM also can be induced by chemical destruction of a portion of the beta cells or surgical removal of part of the pancreas (10). But these models do not resemble T2DM in humans in which the disease is often preceded by obesity. Diet-induced diabetes in C57BL/6J mice is a model of T2DM developed by dietary manipulations in otherwise healthy animals (11, 12). A hope is to detect pathological changes in the pancreas that may represent common conditions associated with T2DM in humans, C57BL/6J mice with diet-induced diabetes were studied.

The pathological changes involved in T2DM with respect to pancreatic islets include hormone secretion dysfunction, proliferation of islet cells during the early phase, and exhaustion of beta cells. Many studies have shown that the exocrine portion of the pancreas secretes proteins that are important in the function and regeneration of beta cells (1–4).
and death of islet cells during the later phase of the disease (13, 14). During the early phase of hyperglycemia, the islets undergo intensive proliferation from ductal progenitor cells and existing islets (15). Loss of an acute phase insulin response and progressive deterioration of beta cell function coupled with peripheral insulin resistance are common in diabetic subjects with chronic hyperglycemia. In T2DM, the toxicity of hyperglycemia, known as glucotoxicity, has both global and tissue-specific effects, including those on the pancreas. However, these functional studies have not provided information on molecular events that underlie the tissue-specific pathological changes.

In this study, we used a proteomic profiling technique; i.e., two-dimensional gel electrophoresis (2-DE) followed by protein identification with mass spectrometry analysis. The advantage of the proteomic approach over microarray or DNA chip analysis is obvious as proteins are detected in the former, whereas mRNAs encoding the proteins are detected in the latter. Use of the technique and the diet-induced diabetes model may help to identify genes and metabolic characteristics in the pancreas that are causal and/or associated with the disease.

EXPERIMENTAL PROCEDURES

Animals—Three-week-old male C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Upon arrival, 50 mice were weaned onto a high fat diet with 26.6% of calories from carbohydrates, 57.6% from fat, and 15.7% from protein (F1850, Bio-Serv, Frenchtown, NJ) (12), while 20 were weaned onto a standard rodent chow containing 60% of calories from carbohydrates, 14% from fat, and 26% from protein (Prolab RMH 3000, PMI Nutrition International, Inc., St. Louis, MO). Mice were housed, up to two per cage, in a temperature-controlled (22 °C) room on a 14-h light, 10-h dark cycle. Food and water were supplied ad libitum. At each time point (2, 4, 8, and 16 weeks on diet), three mice from each group were sacrificed by cervical dislocation. Mice from each group were selected according to their weights and fasting blood glucose and plasma insulin levels. Mice from the high fat diet displaying the following symptoms: overeating, polyuria and polydipsia were selected as diabetic mice, while mice from the normal controls. The pancreata were removed and immediately snap frozen in liquid nitrogen and then stored at −80 °C until further processing. Food and water were supplied ad libitum.

Mouse Weight Measurements—Mice were weighed biweekly during the first 10 h followed by 10 h at 6000 V with a current limit of 16 mA/strip. Following IEF, the strips were equilibrated in a buffer containing 6 M urea, 2% (w/v) SDS, 0.375 M Tris/HCl (pH 8.8), 20% (v/v) glycerol for 15 min before loading for secondary SDS-PAGE analysis. The strips were sealed on the border of the SDS-PAGE gel using 0.5% low melting point agarose gel. Proteins were separated by size in 15% SDS-polyacrylamide gels (20 × 20 cm) at 26 mA/gel with a maximum voltage of 300 V for 9 h at 4 °C. After electrophoresis, the gels were fixed in a solution containing 40% EtOH, 2% acetic acid, 0.0005% SDS overnight followed by washing three times in a buffer of 2% acetic acid and 0.0005% SDS. The gels were stained using a fluorescent dye, SYPRO Orange (1:5000) (Molecular Probes, Eugene, OR), as described previously (21, 22).

Quantitative Analysis of Gel Images—Gel images were captured by a laser-scanning device (FLA-3000G, Fuji). The densities of protein spots, which were normalized by the total densities of all validated and matched spots in a set of gels, were quantitatively compared between diabetic and normal controls using the PDQuest 7.0 program (Bio-Rad). For each time point (2, 4, 8, and 16 weeks on diet), pancreatic protein samples from three diabetic mice and from three control mice were analyzed. Protein “spots” were considered to be differential if the difference between the averages of spot densities from the diabetic mice and the control mice was 2-fold or greater at any time point (23–25).
Differential Pancreatic Proteins in Diabetes

**RESULTS**

**High Fat Diet Induces Obesity**—The weights of the mice on the high fat (HF) diet and their control littermates on the normal, low fat (LF) diet were recorded biweekly from weaning at the age of 3 weeks throughout the course of the study (Fig. 1). Significant differences of body weights between these two groups of mice were observed at 2 weeks and throughout the study.

**Glucose Homeostasis Is Altered by High Fat Diet**—Fasting blood glucose levels in the HF group of mice were significantly higher compared with those in the LF controls at all time points (Fig. 2). At each time point, three obese/diabetic and three control mice were chosen in this study. At the time these mice were sacrificed, their fasting glucose concentrations

---

**Fig. 1.** Body weight profiles for two groups of C57BL/6J mice either on LF or HF diet. Weights were obtained biweekly and plotted for each group. Values are means ± S.E. The means were obtained from all treated animals, which at the start of the study was n = 20 for the LF group and n = 50 for the HF group. Because mice from both groups were sacrificed at 2, 4, 8, and 16 weeks, mouse numbers progressively decreased, and the n values for the nine time points (0, 2, 4, 6, 8, 10, 12, 14, and 16 weeks on diet) were as follows: LF, n = 20, 20, 16, 12, 12, 8, 8, and 8; HF, n = 50, 50, 46, 31, 31, 25, 25, 25, and 25. Spectra, the peaks were calibrated by default and smoothed. All peaks were deisotoped. The Mascot program has the “Peptide Mass Fingerprint Search” engine for a probability-based peptide mass fingerprint database search and the “MS/MS Ions Search” for an MS/MS search. The general parameters for searching are the National Center for Biotechnology Information (NCBI) Database, all species, trypsin digestion, maximum one missed cleavage, fixed carbamidomethylation of Cys, variable modifications of acetyl-N terminus, oxidation-M (methionine), pyro-Glu, and ±50 ppm of peptide mass or parent tolerance. A peptide charge state of 1+ and fragment mass tolerance of ± 0.5 Da were used for the MS/MS ion search. At the time of searching (September 21, 2004), the NCBI nr September 16, 2004 database contained 2,026,219 sequences and 679,922,428 residues.

**Northern Blot Analysis**—Equal amounts of total RNA (15 μg) from different samples were resolved by a 1% agarose gel in 3(N-morpholino)propanesulfonic acid/formaldehyde solution and transferred to a positively charged nylon membrane using the NorthernMax kit (Ambion, Austin, TX) according to the manufacturer’s instructions. All reagents for probe labeling and Northern blot detection were purchased from Roche Applied Science unless stated otherwise. Probe preparation and signal detection were carried out using a DIG labeling and detection kit following the manufacturer’s instructions. Primers (synthesized by Sigma-Genosys, The Woodlands, TX) for probe synthesis were derived from clones that contain mouse Reg2 and Gpx1 cDNAs (Clones BM053708 and BI145182, Open Biosystems, Huntsville, AL). Plasmid DNA was isolated using a miniprep kit (Qiagen, Valencia, CA) according to the instructions of the manufacturer. DIG-labeled probes were synthesized using the Roche Applied Science PCR DIG probe synthesis kit. Labeling and subsequent mRNA signal detection were performed according to the protocols of Roche Applied Science. 5’-AGGGGATAAACAATTTCACACAGG (sense) and 5’-CCCAGTCACGACGTTGTAAAACG (antisense) were used for 700-bp Reg2 probe synthesis. 5’-TGCCGGAGATCAGCGGTCT (sense) and 5’-GGCTCTGAACTTGCAGACAAAG (antisense) were used for 1000-bp Gpx1 probe synthesis. The membranes were hybridized with DIG-labeled probes (50 ng/ml hybridization solution) overnight at 42 °C. The membranes were then washed twice with 2× SSC, 0.1% SDS at room temperature followed by two washes with 0.1× SSC, 0.1% SDS at 68 °C. After treatment in 1× blocking solution, the membranes were incubated with anti-digoxigenin-alkaline phosphatase Fab fragment for 30 min at room temperature and finally detected using CDP-Star chemiluminescent substrate. Images were obtained with VersaDoc (Bio-Rad), and densitometric analysis was then performed using Quantity One software (Bio-Rad).

**Statistical Analysis**—Results are presented as mean ± S.E. Data were analyzed by single factor analysis of variance (Microsoft Excel). Differences were considered statistically significant if p < 0.05.

**RESULTS**

**High Fat Diet Induces Obesity**—The weights of the mice on the high fat (HF) diet and their control littermates on the normal, low fat (LF) diet were recorded biweekly from weaning at the age of 3 weeks throughout the course of the study (Fig. 1). Significant differences of body weights between these two groups of mice were observed at 2 weeks and throughout the study.

**Glucose Homeostasis Is Altered by High Fat Diet**—Fasting blood glucose levels in the HF group of mice were significantly higher compared with those in the LF controls at all time points (Fig. 2). At each time point, three obese/diabetic and three control mice were chosen in this study. At the time these mice were sacrificed, their fasting glucose concentrations

---

**Fig. 2.** Fasting blood glucose levels at different ages for two groups of mice either on LF or HF diets. Means of blood glucose levels for each of the two groups of mice were obtained after an 8-h fasting and plotted. Values are means ± S.E. The means were obtained from all treated animals, which at the start of the study was n = 20 for the LF group and n = 50 for the HF group. Because mice from both groups were sacrificed at 2, 4, 8, and 16 weeks, n values progressively decreased; n = 20, 16, 12, and 8 for the LF group, and n = 50, 46, 31, and 25 for the HF group. Significant difference from control (p < 0.01) was shown at all time points.

**Fig. 3.** Fasting plasma insulin levels at different ages for two groups of mice on either LF or HF diets. Means of insulin levels for the two groups after an 8-h fasting were obtained and plotted. Values are means ± S.E. The means were obtained from all treated animals; n = 20, 16, 12, and 8 for the LF group, and n = 50, 46, 31, and 25 for the HF group. Significant difference from control (p < 0.01) was shown at all time points except for 2 weeks on diet.
Differential Pancreatic Proteins in Diabetes

were 216.1 ± 12.3 and 133.6 ± 4.0 mg/dl for the obese/diabetic mice and the control mice, respectively. The fasting plasma insulin levels in the HF group were significantly higher than those of the control LF group and showed a significant increase throughout the time course of the study (Fig. 3).

Differential Protein Expression as a Consequence of High Fat Diet—Proteins from pancreata of high fat-fed diabetic and control mice were resolved by 2-DE. Of ~450 spots analyzed in the molecular mass range of 5–45 kDa, four protein spots showed a difference of 2-fold or greater when comparing spots from obese/hyperglycemic mice with those from normal controls. The locations of these four spots on a 2-DE gel are shown in Fig. 4. The identifications of these spots by MS analysis are listed in Table I and Table II. Spots A7 and B6 (which were identified as REG1 by MS analysis) had the same two tryptic peptide fragments that were identified by MS/MS analysis (both \( p < 0.05 \)) (Table II). Spots A7 and B6 are predicted to be the same protein. This could be possible via different post-translational modification states. Only one spot (B7, GSHPX1) showed a 2-fold or greater decrease in response to high fat feeding. Table III summarizes these spots and their relative levels at the four different time points studied.

Differential Reg2 and Glutathione Peroxidase Expression by Northern Blot—REG2 and GSHPX1 were the two proteins that showed the most dramatic up-regulation and down-regulation, respectively, in the diabetic mice compared with those of the controls (Table III). The genes encoding these proteins were selected for RNA analysis to check the consistency between protein and RNA levels. Reg2 and Gpx1 RNA levels in the pancreata of diabetic and control mice were determined by Northern blot analysis (Fig. 5). The RNA samples were extracted from two animals that were different from the animals used for protein analysis but possessed similar phenotypic characteristics. Consistent with the protein levels, the Reg2 RNA levels were substantially higher, whereas those of Gpx1 were relatively lower in the pancreas of diabetic mice compared with those in the controls. The average ratios of diabetics/controls as determined by signal densities were 15 and 0.46 for Reg2 and Gpx1, respectively.

DISCUSSION

Diet-induced obesity and diabetes were successfully established in C57BL/6J mice fed a high fat diet. It was unexpectedly observed that some of the mice exhibited hyperglycemia after only 2 weeks on the diet. At each of the four different time points studied, three mice from each group were selected for proteomic analysis based on their weights and fasting glucose and insulin levels that were representative for either obese/diabetic or normal controls.

Several studies have shown that high fat diet and genetic predisposition are critical for the development of hyperinsulinemia and hyperglycemia in these C57BL/6J mice (11, 26, 27). Defects in insulin reaction to glucose stimulation in these mice suggest that they have a genetically determined impairment in their beta cell function that renders them vulnerable to

![Fig. 4. 2-DE image of the pancreas of C57BL/6J mouse fed on LF diet for 8 weeks.](image)

TABLE I

| Spot no. | Protein | Database accession no. | Theoretical pI/Mr | Sequence coverage | PMF probability score | No. of masses (matched/non-matched) |
|----------|---------|------------------------|-------------------|------------------|----------------------|-----------------------------------|
| A7       | REG1    | 6677703                | 6.09/18,519       | 47               | 64\(^d\)             | 6/45                              |
| B6       | REG1    | 6677703                | 6.09/18,519       | 40               | 63\(^d\)             | 6/47                              |
| A6       | REG2    | 6677705                | 5.90/19,407       | 44               | 80\(^d\)             | 6/21                              |
| B7       | GSHPX1  | 121666                 | 6.73/22,282       | 23               | 69\(^d\)             | 5/11                              |

\(^a\) UniGene accession number.

\(^b\) Derived from ExPASy web site (us.expasy.org/). The theoretical signal peptides in REG1 and REG2 are not excluded from the calculations.

\(^c\) Peptide mass fingerprint probability score as defined by Mascot (www.matrixscience.com). Ion score is \(-10 \times \log(p)\) where \(p\) is the probability that the observed match is a random event.

\(^d\) Scores are significant \( (p < 0.05) \).
develop T2DM when environmental factors, such as high fat diet-induced obesity, exaggerate this predisposition (11). Thus, C57BL/6J mice can serve as a valuable animal model mimicking the human condition of obesity-induced diabetes.

To study altered gene expressions associated with a certain disease state, different methodologies can be used. Others have used a microarray-based approach to ascertain gene expression as a function of progression of an individual from a state of obesity to that of T2DM (28). In the results presented here, a proteomic profiling technique, 2-DE analysis coupled with mass spectrometry, was used. This approach enables direct qualitative and quantitative analysis of proteins as the disease develops (21). Also the documented lack of correlation between mRNA levels and protein levels, which may be due to post-transcriptional and translational regulations, necessitates the need for direct protein profiles (21, 25, 29, 30).

We reported three proteins (REG1, REG2, and GSHPX1) that were differentially expressed in the pancreas between normal and obese/diabetic mice. We also observed variations on the magnitude of the difference at various time points for each of the three proteins. We repeated the experiment with another set of mice receiving the same treatment, and similar results were observed.

REG1 and REG2 were two pancreatic proteins that showed an increased level of expression in diabetic C57BL/6J male mice versus controls. Reg genes belong to a gene family that

TABLE II

| Spot no. | Protein name | Sequences of matched peptides by MS (start/end amino acid) | Sequence(s) by MS/MS with ion scorea |
|----------|--------------|------------------------------------------------------------|-------------------------------------|
| A7 REG1  | ISCPEGSNAYSSCYYFTEDR (33–53) | (R)WHWSSGLFLYK(S) Score: 63b (R)ISCPEGSNAYSSCYYFTEDR(L) Score: 99b |
| B6 REG1  | ISCPEGSNAYSSCYYFTEDR (33–53) | (R)WHWSSGLFLYK(S) Score: 86b (R)ISCPEGSNAYSSCYYFTEDR(L) Score: 97b |
| A6 REG2  | INCEPAGANAYSYCYLIEDR (41–61) | (K)WKDENCEAQYSFVCK(F) Score: 39b |
| B7 GSHPX1| YVRPGGEPNFLTEFK (96–112) | Not performedc |

TABLE III

| Spot no. | Protein name | Ratio of averages (diabetic/control)a |
|----------|--------------|-------------------------------------|
| A7 REG1  | 20.0 0.99 0.92 1.5 |  |
| B6 REG1  | 2.2 1.2 0.71 1.1 |  |
| A6 REG2  | 3.1 1.5 1.2 4.6 |  |
| B7 GSHPX1| 0.97 0.67 1.2 0.30 |  |

* MS/MS probability score as defined by Mascot (www.matrixscience.com).

b Significant ion score (p < 0.05).

c The TOF MS data were scored as highly significant in identifying the protein. Also the same protein identification was determined when another MS analysis was performed in a repeated experiment. Also the molecular mass of the spot in the two-dimensional gel that matched the predicted size of the protein was used as supporting data for protein identification.
Differential Pancreatic Proteins in Diabetes

has four subclasses consisting of Reg1 to Reg4 (31, 32). Reg2 and Reg4 are found only in mouse and human, respectively. It is believed that pancreatic stone protein (2, 33–35) and pancreatic thread protein (36) are also encoded by the same human Reg1 gene. In our study, two spots (B6 and A7) with a similar molecular mass but different pI values were identified as Reg1. Because Reg1 is a secreted protein, this pI difference could be due to different glycosylation or other post-translational modifications. However, no definitive post-translational modification could be derived from our mass spectrometry data. The mouse Reg genes are located in a contiguous region in chromosome 6 (37). The mouse has two Reg genes, Reg1 and Reg2, with the mouse Reg1 gene having slightly higher homology to the rat and human Reg1 gene than Reg2 (38).

Several studies have been conducted to locate sites of gene expression for Reg1 and Reg2. Reg1 has been found to be expressed in the exocrine pancreas by acinar cells and secreted into pancreatic ducts (1, 2, 39). Reg1 is also expressed specifically in regenerating and hyperplastic islets but not in normal islets, liver, or brain (40, 41). Low expression of Reg1 has also been found in gastric mucosa, kidney (33), and gallbladder (38). In C57BL/6J mice, Reg1 and Reg2 mRNAs have not been detected in islets of normal mice (42). The question whether the expression of Reg1 and Reg2 in islets of C57BL/6J mice can be induced by the obese/diabetic state remains to be answered. It is likely that the increased expression of Reg1 and Reg2 observed in this study is mainly, if not totally, due to their up-regulated expression in the exocrine pancreas. Future histoimmunological studies will resolve the site(s) of expression.

The expression of Reg1 and Reg2 genes has been previously associated with diabetes. Overexpression of Reg1 and Reg2 in the pancreas of NOD mice at various degrees of diabetogenesis has been found (43–45). In this NOD mouse model, expressions of both Reg1 and Reg2 mRNAs are found to be restricted to acinar cells of the exocrine tissue (45). With Reg1- and Reg2-specific CDNA probes, it has been shown that the increased expression of Reg genes in NOD mice is mainly due to increased expression of Reg2 but not Reg1 (43). Interestingly in normal C57BL/6J mice, when Reg1- and Reg2-specific cDNA probes are used, a decline in the expression of Reg1 but unchanged expression of Reg2 has been observed during the normal aging process (42). These results suggest that the expression of these two non-allelic genes may be differential and that they may have different physiological functions. These results also suggest the importance of studies that discern these two genes as many studies have not separated them because of the cross-reactivity of the Reg RNA probes and the antibody used. In our study with 2-DE, we were able to determine the levels of Reg1 and Reg2 separately. Of four time points in this study, Reg2 showed a 2-fold or greater increase in pancreas in diabetic mice at two time points (2 and 16 weeks on diet), whereas Reg1 showed an increase at one time point (2 weeks on diet). It is interesting to note that Reg2 expression in the pancreas also showed a 2-fold or greater increase in the diabetic state, suggesting the involvement of Reg2 during disease progression.

Several lines of evidence support that Reg1 protein is a stimulator of beta cell proliferation and neogenesis. Administration of Reg protein in 90% pancreatectomized rats results in a remarkable decrease in blood glucose levels and an increase in beta cell mass (3). A similar proliferative effect has also been observed in NOD mice with administration of Reg protein alone or in combination with linomide, an immunomodulatory molecule (4). Transgenic mice that overexpress Reg1 in beta cells exhibit a significant delay in developing diabetes as compared with non-transgenic mice (46). Although Reg1 gene knock-out mice appear phenotypically normal, the average size of islets is significantly smaller than that of wild-type littermates under chemically induced hyperplastic condition (46). It is worth noting that these studies only involve Reg1 not Reg2. However, in the NOD mice study described above, Reg2 but not Reg1 was found to be differentially expressed (43). Both Reg1 and Reg2 were found to be overexpressed in our study, suggesting the biological importance of Reg2. Although Reg2 shows high homology to Reg1, whether Reg2 plays a role similar to that of Reg1 remains to be explored.

It has been suggested that Reg1 protein plays an important role in both beta cell replication from existing islets and neogenesis from ductal cells (47, 48). Beta cells regenerate slowly with a dynamic balance between beta cell replication/neogenesis and apoptosis (15). Beta cells can compensate for insulin resistance or pregnancy by replication and hypertrophy. However, in both type 1 and later stage T2DM, pancreatic beta cells are either destroyed completely or damaged substantially (13).

Islet cells are derived from the epithelial cells of early pancreatic ducts during embryogenesis. In the adult pancreas, neogenesis of islets from ductal cells occurs under both physiological and pathological conditions. Due to its growth-promoting effect on beta cells, the up-regulation of Reg1 gene during disease development suggests that islets undergo active proliferation as a response to hyperglycemia. Thus, the increased expression of Reg1 proteins in diabetic mice may be considered as a defense mechanism and therefore a favorable response. However, the specific role of Reg1 during the progression of the animal from a normal state to that of diabetic still needs to be firmly established.

Another important result was a decreased level of GSHPX1 in the diabetic mice at later stages of T2DM. GSHPX1, a selenium-containing enzyme, is considered the most abundant isoform in the glutathione peroxidase gene family, which consists of at least five genes. It functions as a GSH-dependent enzyme to remove hydrogen peroxide and fatty acid hydroperoxide. It is believed to be ubiquitously expressed in all mammalian tissues with cytosol and mitochondria subcellular localization (49, 50). There is substantial evidence that shows chronic hyperglycemia in diabetes results in overproduction of reactive oxidative species (ROS) and subsequent adverse effects on major molecules and cellular structures, a process
known as glucose toxicity (51–53). The enzymatic antioxidant system that cells utilize to minimize the cellular damages caused by toxic ROS includes various forms of superoxide dismutases, catalase, and glutathione peroxidases.

It has been shown that supraphysiologic glucose concentrations result in high levels of intracellular peroxide concentrations in isolated islets damaging beta cell function (54). These adverse effects can be prevented by transient overexpression of Gpx1 gene in islets suggesting an important antioxidant role of GSHPX1. Overexpression of Gpx1 in the transgenic mice renders hearts more resistant to ischemia reperfusion injury compared with those of control mice (55, 56). The Gpx1-deficient mice are remarkably more sensitive to oxidative stress (57–59). These studies support the role of GPX1 in the protection against oxidative stress and in disease pathogenesis.

Of many tissues in which oxidative stress can cause damage, islets are especially vulnerable. Extremely low levels of gene expression of intrinsic antioxidant enzymes, especially catalase and glutathione peroxidase, in pancreatic islets compared with those in other tissues render islets especially susceptible to ROS-induced damage (60, 61). Down-regulated expression of glutathione peroxidase in pancreas in diabetic mice may result in less capacity to clear ROS. Whereas the demand for antioxidative enzymes is high, this imbalance may contribute to the progressive deterioration of beta cell function in diabetic mice with chronic hyperglycemia.

Using the diet-induced obesity/diabetes mouse model, we discovered three proteins that may play a role in pancreatic dysfunction. Our results suggest that maintenance of the balance between beta cell proliferation and cell death is very important for beta cell function. Also amelioration of the oxidative stress on beta cells may have significant benefit for diabetics.

Acknowledgments—We thank Dr. Douglas T. Kohn for critical reading and comments with the manuscript, Gayle Matheny for conducting the glucose and insulin tests, Dr. Karen Coschigano for providing the pancreas RNA samples, and Dr. Shigeru Okada for collecting some of the tissues used in this study.

* This work was supported in part by DiAthegen LLC, the State of Ohio’s Eminent Scholar Program that includes a gift from Milton and Ohio’s Eminent Scholar Program that includes a gift from Milton and E. G. Kuhl, C. M., and McCubbin, J. A. (1984) Behavioral manipulation of the diabetic phenotype in ob/ob mice. Diabetes 33, 616–618

1. De Reggi, M., Gharib, B., Patard, L., and Stoven, V. (1998) Lithostathine, the rat pancreatic stone protein messenger RNA. Abundant expression in mature exocrine cells, regulation by food content, and sequence identity with the endocrine reg transcript. J. Biol. Chem. 263, 786–791

2. Rouquier, S., Verdier, J. M., Iovanna, J., Dagorn, J. C., and Giorgi, D. (1991) Rat pancreatic stone protein messenger RNA. Abundant expression in mature exocrine cells, regulation by food content, and sequence identity with the endocrine reg transcript. J. Biol. Chem. 266, 786–791

3. Watanabe, T., Yonemura, Y., Yonekura, H., Suzuki, Y., Miyashita, H., Sugiyama, K., Morizumi, S., Unno, M., Tanaka, O., and Kondo, H. (1994) Pancreatic beta-cell replication and amelioration of surgical diabetes by Reg protein. Proc. Natl. Acad. Sci. U. S. A. 91, 3589–3592

4. Gross, D. J., Weiss, L., Reibstein, L., van den Brand, J., Okamoto, H., Clark, A., and Slavin, S. (1998) Amelioration of diabetes in nonobese diabetic mice with advanced disease by linoamide-induced immunoregulation combined with Reg protein treatment. Endocrinology 139, 2369–2374

5. Bjornstorp, P. (1988) Abdominal obesity and the development of noninsulin-dependent diabetes mellitus. Diabetes Metab. Rev. 4, 615–622

6. Surwit, R. S., Feinglos, M. N., Livingston, E. G., Kuhn, C. M., and McCubbin, J. A. (1984) Behavioral manipulation of the diabetic phenotype in ob/ob mice. Diabetes 33, 616–618

7. Kuhn, C. M., Cochrane, C., Feinglos, M. N., and Surwit, R. S. (1987) Examined peripheral responses to catecholamines contributes to stress-induced hyperglycemia in the ob/ob mouse. Pharmacol. Biochem. Behav. 26, 491–495

8. Coleman, D. L. (1978) Obese and diabetes: two mutant genes causing diabetes-obesity syndromes in mice. Diabetologia 14, 141–148

9. Chua, S. C., Jr., Chung, W. K., Wu-Peng, X. S., Zhang, Y., Liu, S. M., Tartaglia, L., and Leibl, R. L. (1996) Phenotypes of mouse diabetes and rat fatty due to mutations in the Ob (leptin) receptor. Science 271, 994–998

10. Portha, B., Blondel, O., Serradas, P., McEvoy, R., Giroux, M. H., Kergoat, M., and Baillie, D. (1989) The rat models of non-insulin-dependent diabetes induced by neonatal streptozotocin. Diabetes Metab. 15, 61–75

11. Wencel, H. E., Smothers, C., Opara, E. C., Kuhn, C. M., Feinglos, M. N., and Surwit, R. S. (1995) Impaired second phase insulin response of diabetes-prone C57BL/6J mouse islets. Physiol. Behav. 57, 1215–1220

12. Surwit, R. S., Kuhn, C. M., Cochrane, C., McCubbin, J. A., and Feinglos, M. N. (1998) Diet-induced type II diabetes in C57BL/6J mice. Diabetes 37, 1163–1167

13. Butler, A. E., Janson, J., Bonner-Weir, S., Ritzel, R., Rizza, R. A., and Butler, P. C. (2003) Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. Diabetes 52, 102–110

14. Weir, G. C., Laybutt, D. R., Kaneto, H., Bonner-Weir, S., and Sharma, A. (2005) Beta-cell adaptation and compensation during the progression of diabetes. Diabetes 50, Suppl. 1, S154–S159

15. Bonner-Weir, S. (2001) Beta-cell turnover: its assessment and implications. Diabetes 50, Suppl. 1, S20–S24

16. Chevallet, M., Santoni, V., Poinas, A., RouQUIE, D., Fuchs, A., Kieffer, S., Rossignol, M., Lunardi, J., Garin, J., and Rabilloud, T. (1998) New zwiterionic detergents improve the analysis of membrane proteins by two-dimensional electrophoresis. Electrophoresis 19, 1901–1909

17. Chomczynski, P., and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162, 156–159

18. Galvani, M., Hamdan, M., Herbert, B., and Righetti, P. G. (2001) Alkylation kinetics of proteins in preparation for two-dimensional maps: a matrix assisted laser desorption/ionization-mass spectrometry investigation. Electrophoresis 22, 1990–1994

19. Herbert, B., Galvani, M., Hamdan, M., Olivieri, E., MacCarthy, J., Pedersen, S., and Righetti, P. G. (2001) Reduction and alkylation of proteins in preparation of two-dimensional map analysis: why, when, and how? Electrophoresis 22, 2046–2057

20. Gorg, A., Postel, W., and Gunther, S. (1988) The current state of two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis 9, 531–546

21. Kopchick, J. J., Ut, E. O., Kohn, D. T., Keidan, G. M., Oiu, L., and Okada, S. (2002) Perspective: proteomics—see “spots” run. Endocrinology 143, 1990–1994

22. Malone, J. P., Radabaug, M. R., Leimgruber, R. M., and Gerstenecker, G. S. (2001) Practical aspects of fluorescent staining for proteomic applications. Electrophoresis 22, 919–932

23. Cho, Y. M., Bae, S. H., Choi, B. K., Cho, S. Y., Song, C. W., Yoo, J. K., and Paik, Y. K. (2003) Differential expression of the liver protein in senescent accelerated mice. Proteomics 3, 1883–1894

24. Page, M. J., Amess, B., Townsend, R. R., Parekh, R., Herath, A., Brusten, L., Zvelebil, M. J., Stein, R. C., Waterfield, M. D., Davies, S. C., and O’Hare, M. J. (1999) Proteomic definition of normal human luminal and myoepithelial breast cells purified from reduction mammoplasties. Proc. Natl. Acad. Sci. U. S. A. 96, 12589–12594
Differential Pancreatic Proteins in Diabetes

25. Huber, M., Bahr, I., Kratzschmar, J. R., Becker, A., Muller, E. C., Donner, P., Pohlenz, H. D., Schneider, M. R., and Sommer, A. (2004) Comparison of proteomic and genomic analyses of the human breast cancer cell line T47D and the antitestrogen-resistant derivative T47D-r. *Mol. Cell. Proteomics* **3**, 43–55.

26. Surwit, R. S., Feinglos, M. N., Rodin, J., Sutherland, A., Petro, A. E., Opara, E. C., Kuhn, C. M., and Reubert-Scrive, M. (1999) Differential effects of fat and sucrose on the development of obesity and diabetes in C57BL/6J and A/J mice. *Metabolism* **44**, 645–651.

27. Surwit, R. S., Seldin, M. F., Kuhn, C. M., Cochrane, C., and Feinglos, M. N. (1991) Control of expression of insulin resistance and hyperglycemia by different genetic factors in diabetic C57BL/6J mice. *Diabetes* **40**, 82–87.

28. de Fourmestraux, V., Neubauer, H., Poussin, C., Farmer, P., Falquet, L., Burcelin, R., Delorennz, M., and Thorens, B. (2004) Transcript profiling suggests that differential metabolic adaptation of mice to a high fat diet is associated with changes in liver to muscle lipid fluxes. *J. Biol. Chem.* **279**, 50743–50753.

29. Gygi, S. P., Rochon, Y., Franca, B. R., and Aebersold, R. (1999) Correlation between protein and mRNA abundance in yeast. *Mol. Cell. Biol.* **19**, 1720–1730.

30. MacKay, V. L., Li, X., Flory, M. R., Turcott, E., Law, G. L., Serikawa, K. A., Xu, X. L., Lee, H., Goodlett, D. R., Aebersold, R., Zhao, L. P., and Morris, D. R. (2004) Gene expression analyzed by high-resolution state array analysis and quantitative proteomics: response of yeast to mating pheromone. *Mol. Cell. Proteomics* **3**, 478–489.

31. Hartupee, J. C., Zhang, H., Bonaldo, M. F., Soares, M. B., and Dieckgraefe, B. K. (2001) Isolation and characterization of a CDNA encoding a novel member of the human regenerating protein family: Reg IV. *Biochem. Biophys. Acta* **1518**, 287–293.

32. Okamoto, H. (1999) The Reg gene family and Reg proteins: with special attention to the regeneration of pancreatic beta-cells. *J. Hepatobiliary Pancreat. Surg.* **6**, 254–262.

33. Watanabe, T., Yonekura, H., Terazono, K., Yamamoto, H., and Okamoto, H. (1998) Complete nucleotide sequence of human reg gene and its expression in normal and tumoral tissues. The reg protein, pancreatic stem protein, and pancreatic thread protein are one and the same product of the gene. *J. Biol. Chem.* **265**, 7432–7439.

34. De Caro, A. M., Bonicel, J. J., Rouimi, P., De Caro, J. D., Sarles, H., and Rovery, M. (1987) Complete amino acid sequence of an immunoreactive form of human pancreatic stem protein isolated from pancreatic juice. *Eur. J. Biochem.* **168**, 201–207.

35. De Caro, A. M., Adrich, Z., Fournet, B., Capon, C., Bonicel, J. J., De Caro, J. D., and Rovery, M. (1989) N-terminal sequence extension in the glycosylated forms of human pancreatic stem protein. The 5-oxoproline N-terminal chain is O-glycosylated on the 5th amino acid residue. *Biochim. Biophys. Acta* **984**, 281–284.

36. Grandi, J., Carlsson, J., Breuer, A. W., Margolies, M. N., Warshaw, A. L., and Wands, J. R. (1985) Isolation, characterization, and distribution of an unusual pancreatic secretory protein in human low secretory pancreatitis. *J. Clin. Invest.* **76**, 2115–2126.

37. Abe, M., Nata, K., Akiyama, T., Shervani, N. J., Kobayashi, S., Tomioka-Kumagai, T., Ito, S., Takasawa, S., and Okamoto, H. (2000) Identification of a novel Reg family gene in a 75 kilobase mouse genomic region. *Mol. Cell. Biol.* **20**, 9943–9953.

38. Forme, S., Takanabe, M., Engelmann, D. T., Engelmaier, R. M., Schley, J. A., Maulik, N., Ho, Y. S., Oberley, T. D., and Das, D. K. (1996) Transgenic mice overexpressing glutathione peroxidase are resistant to myocardial ischemia-reperfusion injury. *J. Mol. Cell. Cardiol.* **28**, 1759–1767.

39. de Haan, J. B., Bladier, C., Griftiths, P., Kelner, M., O’Shea, R. D., Cheung, N. S., Bronson, R. T., Silvestro, M. J., Wild, S., Zheng, S. S., Beart, P. M., Hertzog, P. J., and Kola, I. (1998) mice with a homozygous null mutation in the hypoxia inducible factor alpha subunit (HIF-1alpha) develop normally, but have defects in erythropoiesis and renal tubular hypoxia response. *Nat. Genet.* **20**, 112–117.

40. Dayal, S., Brown, K. L., Weydert, C. J., Oberley, L. W., Amri, E., Bottiglieri, T., Faraci, F. M., and Lentz, S. R. (2002) Deficiency of glutathione peroxidase-1 sensitizes hyperhomocysteinemic mice to endothelial dysfunction. *Arterioscler. Thromb. Vasc. Biol.* **22**, 950–956.

41. Forgione, M. A., Weiss, N., Heydrick, S., Cap, A., Klings, E. S., Bierl, C., de Haan, J. B., Bladier, C., Griffiths, P., Kelner, M., O’Shea, R. D., Cheung, N. S., Bronson, R. T., Silvestro, M. J., Wild, S., Zheng, S. S., Beart, P. M., Hertzog, P. J., and Kola, I. (1998) Mice with a hypoxia inducible factor alpha subunit (HIF-1alpha) null mutation are viable and display a normal phenotype. *Nat. Genet.* **20**, 112–117.