Effect of Rosiglitazone on the Differential Expression of Diabetes-associated Proteins in Pancreatic Islets of C57Bl/6 *lep*/*lep* Mice*

Jean-Charles Sanchez‡§, Véronique Converset‡, Anna Nolan¶, Gerhard Schmid‡, Steven Wang¶, Manfred Heller‡, Matthew V. Sennitt¶, Denis F. Hochstrasser‡, and Michael A. Cawthorne¶

The insulin sensitizer drug, rosiglitazone, has been shown to have a protective effect on pancreatic islet cell structure and function in animal models of type 2 diabetes. The identification of new molecular targets associated both with islet cell dysfunction and protection is a crucial research goal. In the present study, a proteomics approach has been used to identify such targets. Obese C57Bl/6J *lep*/*lep* mice and lean littermates were given the insulin sensitizer drug BRL49653, rosiglitazone. It normalized the impaired glucose tolerance in *lep*/*lep* mice but had no significant effect on glucose tolerance in the lean mice. Pancreatic islet polypeptides were arrayed by a two-dimensional gel electrophoresis system that separated more than 2500 individual spots. Three overexpressed and six underexpressed proteins were significant ($p < 0.05$) between *lep*/*lep* and lean mice, and four were modulated significantly ($p < 0.05$) by the rosiglitazone treatment of the obese mice. The identity of these differentially expressed proteins was made using mass spectrometric analysis and provided evidence that differential expression of actin-binding proteins may be an important aspect of defective islet function. Rosiglitazone increased carboxypeptidase B expression in both *lep*/*lep* and normal mice suggesting that this might be an independent effect of rosiglitazone that contributes to improved insulin processing. ***Molecular & Cellular Proteomics 1: 509–516, 2002.***

Type 2 diabetes is a complex metabolic disease involving defects in both insulin secretion and insulin action and is influenced by a range of genetic and environmental factors (1). As in other polygenic diseases, including cancers and cardiovascular diseases, advances in molecular genetic approaches have made it possible to start to identify susceptibility genes of type 2 diabetes, but so far the search for candidate genes has been largely negative (2) probably as a result of redundancy and compensatory mechanisms (3). Linear genetic strategies are thus not sufficient to predict specific phenotypes, and more global approaches that combine genomic and proteomic investigation are required.

Whether insulin resistance or insulin secretion defects are primary in the development of type 2 diabetes has been a highly debated topic, and there is no consensus. However, it is clear that overt type 2 diabetes only occurs when the insulin output from the pancreatic islet fails to match the insulin requirement as a result of the insulin resistance (4).

Current therapies targeted to the pancreatic islet are agents such as sulfonylureas that increase meal-induced insulin secretion. However, it is clear that although these agents can be effective in the short term they can also accelerate the progression of the diabetic state (5). There is a growing opinion that new agents are required that increase the pancreatic islet cell mass and/or enhance the production of insulin by the islet cell (6). Recently, insulin sensitizer drugs, such as BRL49653 (rosiglitazone), have become available. These agents increase insulin sensitivity of adipose tissue, skeletal muscle, and liver (7) and reduce the insulin requirement. BRL49653 has also been shown to prevent or alleviate islet hyperplasia in Zucker *fa*/*fa* rats (8), and human studies provide evidence of islet cell protection (9). Animal studies have also shown that islet cell mass might affect significantly the susceptibility to develop diabetes. Thus, C57Bl/6 mice have twice the islet cell mass of C57Bl/Ks mice (10), and the latter mice are significantly more susceptible to diabetes induced by chemical damage (11) or as a result of the acquisition of the obesity or obesity receptor gene mutations *lep* or *lepR*, previously known as *ob* and *db* (12).

In the present study, we have used two separate phenotypic paradigms to undertake a systematic identification of proteins associated with anatomical and functional abnormalities of pancreatic islets. First we have used C56Bl/6J *lep*/*lep* mice and lean littermates that have been treated with the insulin sensitizer BRL49653. Because this agent normalizes glucose tolerance and insulin sensitivity in the *lep*/*lep* mice but has little effect in the littermates (7), it follows that islet proteins that are expressed differentially between *lep*/*lep* and lean C57Bl/6J mice and whose expression is modulated by

From the ‡Geneva Proteomics Center, Central Clinical Chemistry Laboratory, Geneva University Hospital, 1211 Geneva 14, Switzerland and the ¶Clore Laboratory, School of Science, University of Buckingham, Buckingham MK18 1EG, United Kingdom

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BRL49653 only in the *lep/lep* mice are disease-associated proteins. Second, we have examined the differential expression of islet proteins in C57Bl/6 and C57Bl/Ks mice to identify proteins potentially associated with low islet cell mass.

EXPERIMENTAL PROCEDURES

Reagents and Apparatus—All reagents and apparatus used have been described in detail elsewhere (13). Mice were obtained from Jackson Laboratories, Bar Harbor, ME.

Mice Treatment—All mice used in this study were 8-week-old females, and they were housed on a 12-h light cycle (light turned on at 08:00) at 21 °C and given food (rat and mouse standard diet; B+K Universal, Hull, United Kingdom) and water *ad libitum*. Each group of mice had its food consumption measured daily. Lean ($n = 4$) and obese ($n = 4$) control C57Bl/6J *lep/lep* mice were given powdered diet. Lean ($n = 4$) and obese ($n = 4$) treated C57Bl/6J *lep/lep* mice were given BRL49653, rosiglitazone (7) (Dextra Laboratories, Readings Berks, United Kingdom) by dietary admixture at the rate of 10 mg/kg diet for 7 days. After an overnight fast, mice were given glucose (3.0 g/kg body weight, per os). Blood from the tail (20 μl) was taken every 30 min (−30 min to +180 min after glucose was given). Blood glucose concentrations were measured as described previously (14). All animal studies were approved by the local ethical review committee and were carried out in accordance with United Kingdom government regulations and NIH guidelines on the care and welfare of laboratory animals.

Pancreatic Islet Preparations and Solubilization—Mice were anesthetized with 50% hyponovel and 50% hyponorm and then killed with carbon dioxide gas. The pancreas was removed, and pancreatic islets were isolated by collagenase digestion at 37 °C using a physiological saline solution supplemented with 1 mM $CaCl_2$, 4 mM glucose and equilibrated with CO_2:O_2 (5%:95%), pH 7.4 (15). Islets were hand-picked using a binocular microscope, washed, microdissected to remove residual acinar material, and finally snap-frozen in liquid nitrogen and stored at −80 °C until analysis. 200 (analytical gels) or 1,000 (preparative gels) islets were mixed with 60 μl of a solution containing urea (8 M), CHAPS[1] (4% w/v), Tris (40 mM), dithiothreitol (65 mM), SDS (0.05% w/v), and a trace of bromphenol blue. The whole final diluted sample was loaded in a cup at the cathodic end of the IPG gels.

Two-dimensional Gel Electrophoresis—A commercial sigmoidal IPG (18 cm nonlinear from Amersham Biosciences) going from pH 3.5 to 10.0 was used for first-dimensional separation (16, 17). After equilibration, the IPG gel strips were transferred for the second dimension onto vertical gradient slab gels (9–16% T, 2.6% C) and run with the Laemmli-SDS-discontinuous system (18–20). Protein detection was achieved using a sensitive ammoniacal silver stain (16, 21) or Coomassie Brilliant Blue R-250 (0.1% w/v) and methanol (50% v/v) for 30 min. Destaining was done in a solution containing methanol (40% v/v) and acetic acid (10% v/v).

Image Acquisition and Data Analysis—Duplicate gels, which had a reproducibility of ±45% for the volume of any of the 2528 detected spots, were run. Gels were scanned using a laser densitometer (Amersham Biosciences). The 2-DE image computer analysis was carried out using the MELANIE 3 software package (GeneBio, Geneva, Switzerland) (22). Spots were detected and quantified automatically. The optical density, the area, and the volume were computed and related directly to protein concentration. The relative optical density and relative volume were also calculated to correct for differences in gel staining. Differential analysis (>200%) and Student's *t* test ($p < 0.05$) using the relative volume of each spot (>0.04%) allowed the detection of significantly over- and underexpressed polypeptides with a minimum ratio of two.

Protein Identification by Mass Spectrometry—The identity of the differentially expressed proteins was made using mass spectrometric analysis including peptide mass fingerprinting analysis and then peptide fragmentation for their validation. Coomassie Blue 2-DE spots were destained with 100 μl of 30% acetonitrile in 50 mM ammonium bicarbonate at 37 °C for 45 min. The supernatant was discarded, and the gel spots were dried in a SpeedVac for 30 min. The gel spots were rehydrated with 25 μl of a solution containing 0.2 μg of porcine trypsin and 50 mM ammonium bicarbonate for 2 h at 35 °C. Then the gel spots and supernatant were dried in a SpeedVac for 30 min, rehydrated with 20 μl of H_2O for 30 min at 35 °C, and dried again for 30 min. 5 μl of a solution containing 50% acetonitrile and 0.1% trifluoroacetic acid was added to the spots and sonicated for 10 min.

Protein Identification by Peptide Mass Fingerprinting—1 μl of the supernatant was loaded in each well of a 96-well matrix-assisted laser desorption ionization target plate. The samples were air-dried. Then 2 μl of a solution containing 4 mg/ml of α-cyano-4-hydroxycinnamic acid, 50% acetonitrile, and 0.1% trifluoroacetic acid was added on each well and air-dried. The peptide mixtures were analyzed by a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (Applied Biosciences Voyager Elite MALDI-TOF-MS) with a nitrogen laser (337 nm) and operated in reflectron-delayed extraction mode. Protein identification was carried out using SmartIdent (www.expasy.ch/sprot/SmartIdent.html). It is a tool that allows the identification of proteins using pI, molecular weight, and peptide mass fingerprinting data. Experimentally measured, user-specified peptide masses were compared with the theoretical peptides calculated for all proteins in the SWISS-PROT/TREMBL databases.

Protein Identification by Peptide Fragmentation—4 μl of the supernatant was desalted by a liquid reversed-phase chromatography system with C18 (75-μm diameter and 10-cm-long) microcolumns. Peptides were applied by nanoflow (in-house nanospray) sample introduction to a tandem mass spectrometer that consists of two quadrupoles and an orthogonal time of flight tube (Q-TOF-MS) from MicroMass (Manchester, United Kingdom). Fragment ion spectra were interpreted with the SEQUEST database search (ThermoFinnigan, San Jose, CA) and compared with theoretical ones from different databases such as SWISS-PROT, TREMBL, and/or expressed sequence tag databases.

RESULTS

Oral Glucose Tolerance Test—The obese C56Bl/6J *lep/lep* strain shows hyperphagia, insulin resistance, glucose intolerance, and fasting hyperglycemia (23) relative to its lean littermates. To check the efficacy of rosiglitazone on blood glucose concentration, an oral glucose tolerance test was performed on the four groups of mice (*i.e.* lean control, obese (*lep/lep*) control, lean treated, and obese (*lep/lep*) treated). Fig. 1 shows that the area under the blood glucose concentration curve of the obese control group is much greater than the lean control. Treatment of the obese group with rosiglitazone normalized the glucose tolerance, whereas treatment of the lean group had no further effect. These results validated that rosiglitazone improves the glycemia of the *lep/lep* group and does not provoke hypoglycemia in the lean littermates.

2-DE Gel Separation and Identification of Proteins—Mouse islets were arrayed on the 2-DE gel system, and proteins were

[1] The abbreviations used are: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; 2-DE, two-dimensional gel electrophoresis; IPG, immobilized pH gradient.

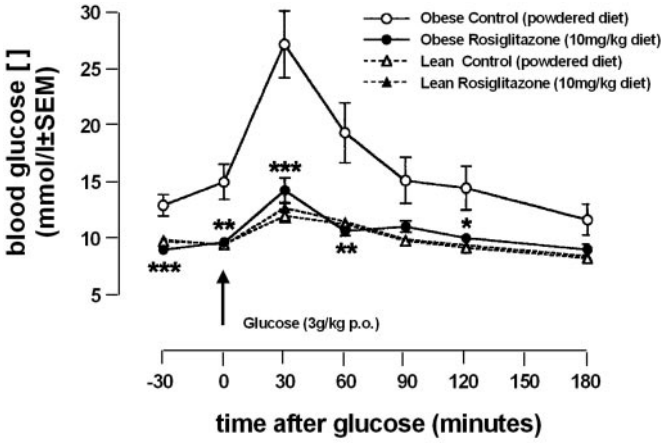

FIG. 1. **Oral glucose tolerance test.** Lean ($n = 4$) and obese ($n = 4$) control C57Bl/6J *lep/lep* mice were given powdered standard diet. Lean ($n = 4$) and obese ($n = 4$) treated C57Bl/6J *lep/lep* mice were given BRL49653, rosiglitazone (19), by dietary admixture at the rate of 10 mg/kg diet for 7 days. After an overnight fast, mice were given glucose (3.0 g/kg body weight, per os), and blood (20 μl) was taken from the tail every 30 min (−30 min to +180 min after glucose was given). Blood glucose concentrations (mmol/liter) were measured by the glucose oxidase method. Results are given as mean ± S.E. Significant differences between glucose concentrations in *lep/lep* mice given rosiglitazone relative to *lep/lep* controls are indicated by *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

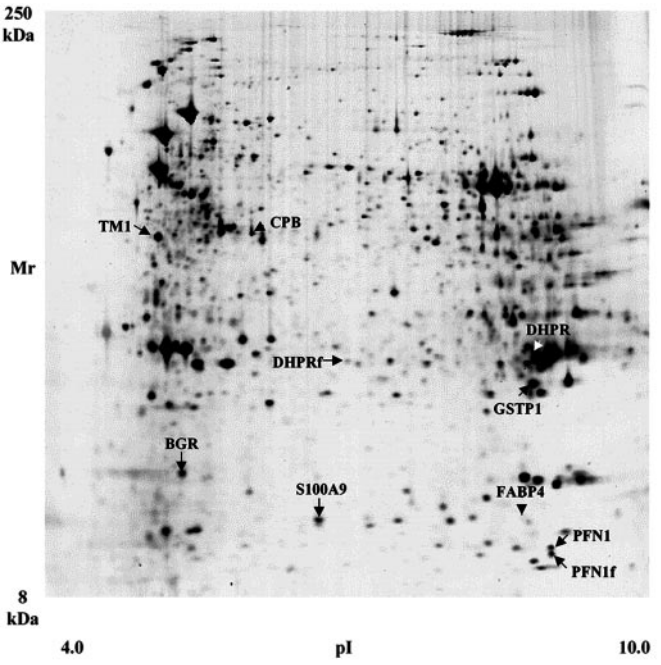

FIG. 2. **Silver-stained mouse islet of Langerhans 2-DE image.** 200 islets were loaded on an IPG gel (3.5–10 nonlinear IPG, 18 cm). The second dimension was a vertical gradient slab gel (9–16% T). The gene names mark the location of the corresponding differentially expressed proteins. *TM1*, tropomyosin isoform 1; *DHPR*, dihydropteridine reductase; *DHPRf*, dihydropteridine reductase fragment; *GSTP1*, glutathione *S*-transferase P1; *BGR*, β-granin; *S100A9*, calgranulin B; *FABP4*, adipocyte fatty acid-binding protein; *PFN1*, profilin; *PFN1f*, profilin fragment; *CPB*, carboxypeptidase B.

identified by mass spectrometric analysis. From the 2528 detected spots corresponding approximately to 1500 proteins and representing only 10% of the whole islet proteome, 63 have been identified that correspond to 44 protein entries. The data have been established in the mouse SWISS-2DPAGE database (24). The database can be found on the ExPASy server (www.expasy.ch/cgi-bin/map2/def?ISLETS_MOUSE). Fig. 2 shows the 2-DE gel electrophoresis image of mouse islets of Langerhans with differentially expressed protein locations marked.

Changes of Protein Expression between lep/lep Mouse Islets and Lean Littermates—Image analysis allowed the detection and identification (Fig. 3) of six underexpressed (4-fold reduction in tropomyosin isoform 1, 10-fold reduction of dihydropteridine reductase and dihydropteridine reductase fragment, 4-fold reduction in glutathione *S*-transferase P1, 4-fold reduction of β-granin, and 3-fold reduction of calgranulin B) and three overexpressed polypeptides (3-fold increase in adipocyte fatty acid-binding protein and 4-fold increase of profilin and profilin fragment) in obese controls relative to lean mice ($p < 0.05$).

Changes of Protein Expression Induced by Treatment with BRL49653—Of the nine polypeptides differentially expressed between *lep/lep* mouse islets and lean littermates, four were modulated significantly ($p < 0.05$) after treatment of the *lep/lep* mice, but not in the lean littermates, with BRL49653. These (tropomyosin isoform 1, adipocytes fatty acid-binding protein, profilin, and profilin fragment) had their expression level altered toward the level in the lean littermates (Fig. 4). In addition, BRL49653 increased carboxypeptidase B precursor protein expression in both *lep/lep* and normal mice (Fig. 5).

Differential Polypeptide Expression between C57Bl/6 and C57Bl/Ks Mice—The yield of islets from lean C57Bl/Ks mice was on average only 50% of the yield from C57Bl/6 mice, and the Ks islets were smaller, in agreement with the studies of Swenne and Andersson (10). Of the 31 polypeptides found to be differentially expressed between these two genotypes (data not shown), two of the proteins (tropomyosin and profilin) had similar levels of expression in the C57Bl/Ks mice to that in the C57Bl/6 *lep/lep* mice (Fig. 6).

DISCUSSION

The obese C57Bl/6J *lep/lep* mouse strain shows obesity, hyperphagia, insulin resistance, glucose intolerance, and mild fasting hyperglycemia (23). Hyperplasia and hypertrophy of β-cell mass are also present (25). The obese phenotype results from a nonsense mutation in codon 105 of the *ob* gene located in chromosome 6 (26). The *ob* gene is expressed mainly in white adipose tissue, and its mutation leads to a failure to secrete the gene product into the circulation. The gene product, leptin ("leptos" in Greek means thin), acts on the hypothalamus to regulate appetite (27). However, leptin receptors (product of the *db* gene) are also present in a variety

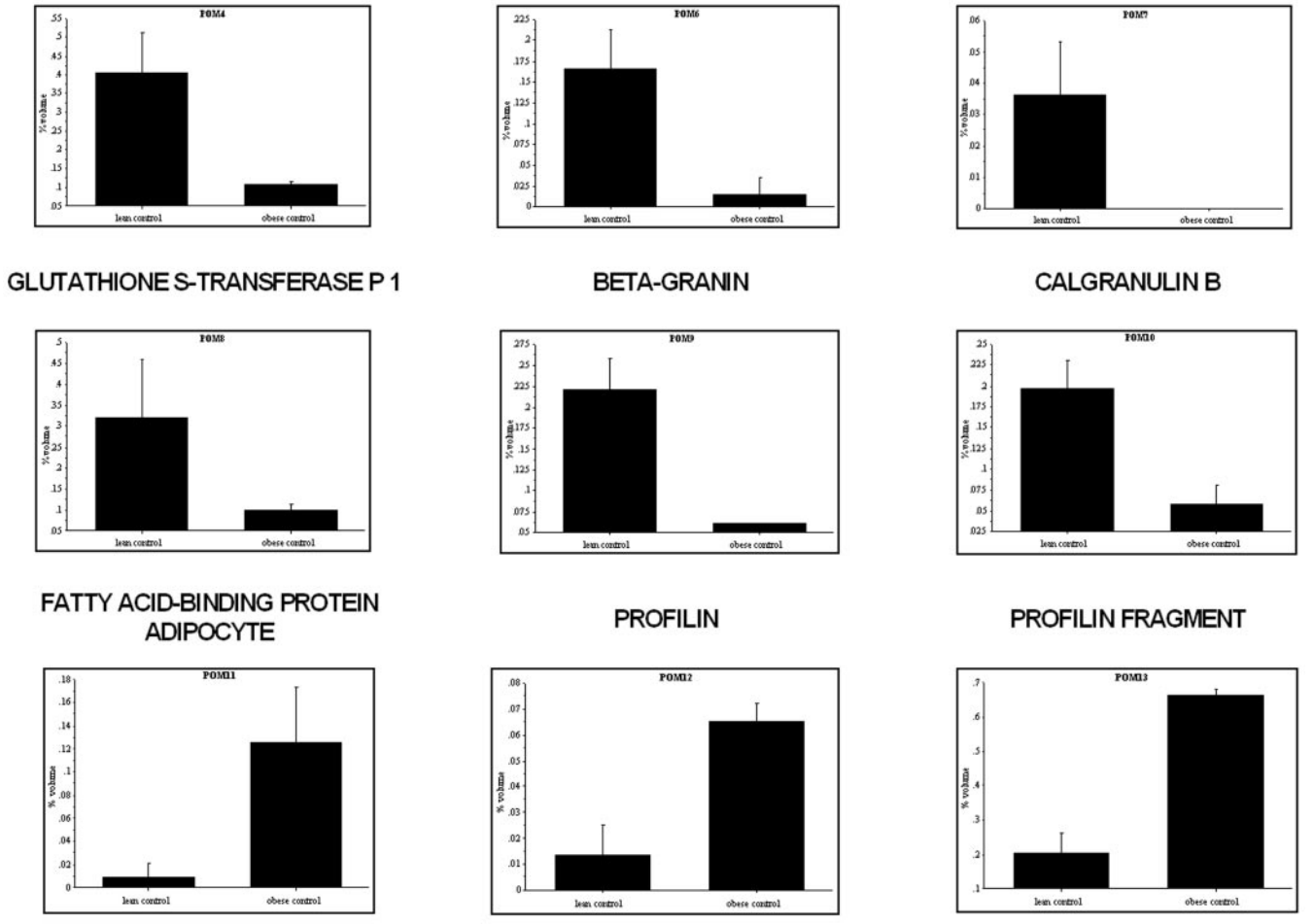

FIG. 3. **Bar charts representing the relative spot volume detected by a differential expression analysis.** Six underexpressed (tropomyosin isoform 1, dihydropteridine reductase, dihydropteridine reductase fragment, glutathione *S*-transferase P1, β-granin, and calgranulin B) and three overexpressed polypeptides (adipocyte fatty acid-binding protein, profilin, and profilin fragment) were significant ($p < 0.05$) between obese control ($n = 4$) and lean control mice ($n = 4$).

of tissues (28) including pancreatic islet cells, and leptin affects insulin secretion (29). The hyperplasia and hypertrophy of the β-cell mass in C57Bl/6J *lep/lep* mice is not solely due to the lep mutation, because when the mutation is expressed on the C57Bl/Ks background, islet cells become damaged, and the mice develop frank diabetes (12). Other studies have shown fundamental differences in islet cell mass between wild-type C57Bl/6 and C57Bl/Ks mice (10).

Recently it has been shown that, in addition to its primary effect on insulin sensitivity, rosiglitazone increases the insulin content of the pancreas in diabetic rats, as well as the number and total mass of islet cells (8, 9). Whether this is a direct effect of rosiglitazone through activation of a peroxisome proliferator-activated receptor γ-mediated pathway in islets or an indirect result of normalizing the glycemia is not known, but the molecular changes that are associated with this islet cell remodeling are potentially important to the long term efficacy of rosiglitazone. In the current study, nine significant ($p < 0.05$) differentially expressed polypeptides were detected between *lep/lep* and control mice, and four of these were modulated by rosiglitazone treatment only in *lep/lep* mice.

Of the differentially expressed proteins not modulated by rosiglitazone, dihydropteridine reductase (and a fragment) were reduced 10-fold in islets from *lep/lep* mice. Dihydropteridine reductase converts dihydrobiopteridin (BH_2) to tetrahydrobiopteridin (BH_4) in a NADH-mediated reaction. BH_4 is an essential molecule in the synthesis of serotonin and catecholaminergic neurotransmitters. As demonstrated by Masiello *et al.* (30), in the brain of streptozotocin-induced diabetic rats, its deficiency potentially results from reduced neurotransmitter synthesis in islets. Glutathione *S*-transferase was reduced 4-fold in *lep/lep* mice, as it was also in white adipose tissue.[2] Glutathione *S*-transferase has also been found to be reduced in liver of *lep/lep* mice, and streptozoto-

[2] J.-C. Sanchez, V. Converset, A. Nolan, G. Schmid, S. Wang, M. Heller, M. V. Sennitt, D. F. Hochstrasser, and M. A. Cawthorne, unpublished results.

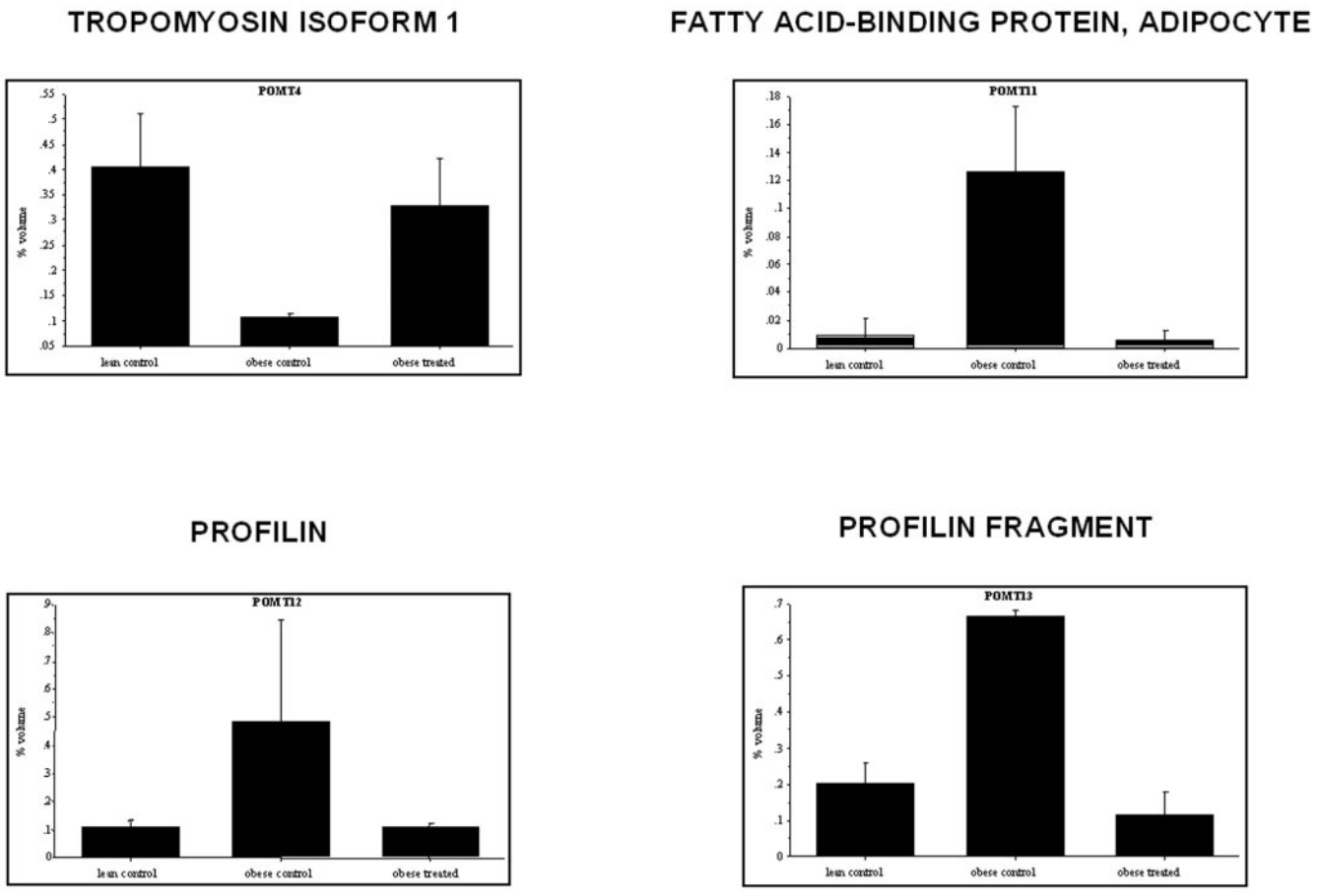

FIG. 4. **Bar charts representing the relative spot volume detected by a differential expression analysis.** Four differentially expressed proteins (tropomyosin isoform 1, adipocyte fatty acid-binding protein, profilin, and profilin fragment) were modulated in obese models ($n = 4$) to the level in lean control ($n = 4$) after rosiglitazone treatment ($p < 0.05$).

cin-induced diabetic rats (31), and in the placenta of overt diabetic patients (32). Reactive intermediates generated through metabolism are detoxicated by several systems. One mechanism is the conjugation of biologically active electrophiles with endogenous tripeptide glutathiones leading to the final formation and excretion of mercapturic acid. The reaction is catalyzed by glutathione *S*-transferase (33). The reasons for this change in expression in islets are not known, but it could result in decreased ability to undertake some detoxification reactions.

Chromogranin A is a member of the granin family of acidic secretory glycoproteins that are expressed in endocrine and neuroendocrine cells. Chromogranin A is cleaved to several peptides including chromostatin, pancreastatin, and parastatin. They act mainly to inhibit hormone and neurotransmitter release in an autocrine or paracrine manner (34). Proteolytic cleavage of chromogranin A gives also rise to β-granin. This N-terminal polypeptide product, which has no known function, was expressed 4-fold lower in islets of *lep*/*lep* mice than in islets from controls. Calgranulin B, which is a member of the S100 family of calcium-binding proteins (35), was 3-fold lower in *lep*/*lep* islets. This could be involved in the abnormal Ca^{2+} signaling in islet cells and affects tyrosine-specific kinases and the organization of the β-cell cytoskeleton.

Of the differentially expressed proteins modulated by rosiglitazone, the adipocyte form of fatty acid-binding proteins was 3-fold higher in islets from *lep*/*lep* mice than in their lean counterparts. Fatty acid-binding proteins are present in a number of tissues. Fatty acids are known to have a profound effect on insulin secretion from islets. Thus acutely fatty acids augment glucose-induced insulin secretion (36), whereas chronic exposure to fatty acids results in an attenuation of glucose-induced insulin secretion (37). The accumulation of fatty acids in islets of Zucker diabetic fatty rats has been associated with deterioration in islet cell responsiveness (38). In the present study, rosiglitazone treatment lowered the expression level in the *lep*/*lep* mice to the level in controls. Rosiglitazone is known to have significant effects on fatty acid metabolism, and reduction in circulating non-esterified fatty acids is an early event. Thus rosiglitazone treatment reduces the exposure of islet cells to fatty acids, and hence the change in binding protein may merely reflect changes in fatty acid exposure. Tropomyosin is an actin-binding protein regulating calcium-dependent cell contraction (39), whereas profilin af-

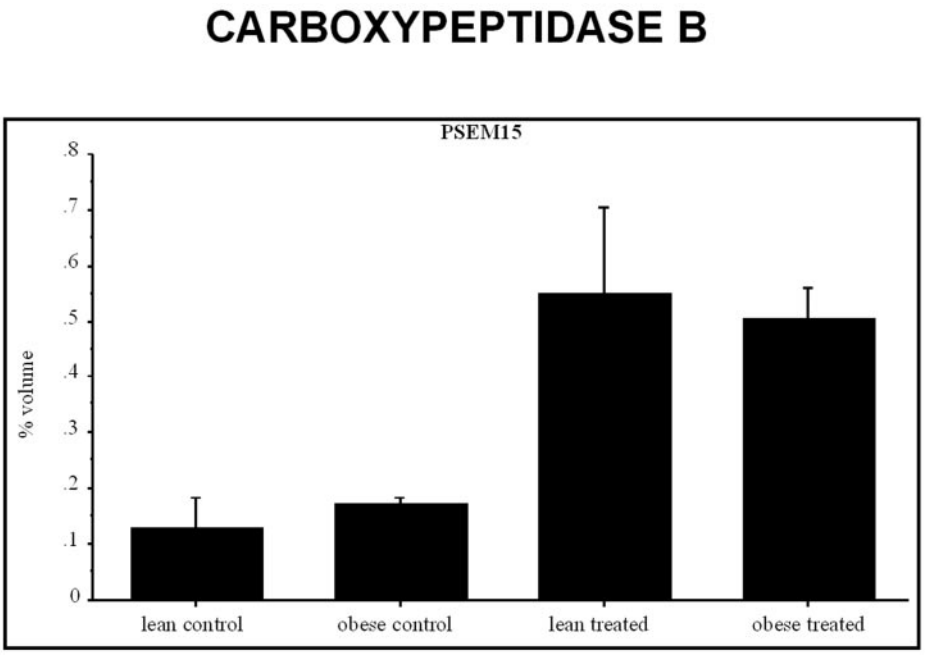

FIG. 5. **Bar chart representing the relative spot volume detected by a differential expression analysis.** Overexpression of carboxypeptidase B precursor protein in obese and lean models ($n = 4$) after rosiglitazone treatment relative to obese and lean controls ($n = 4$) ($p < 0.05$).

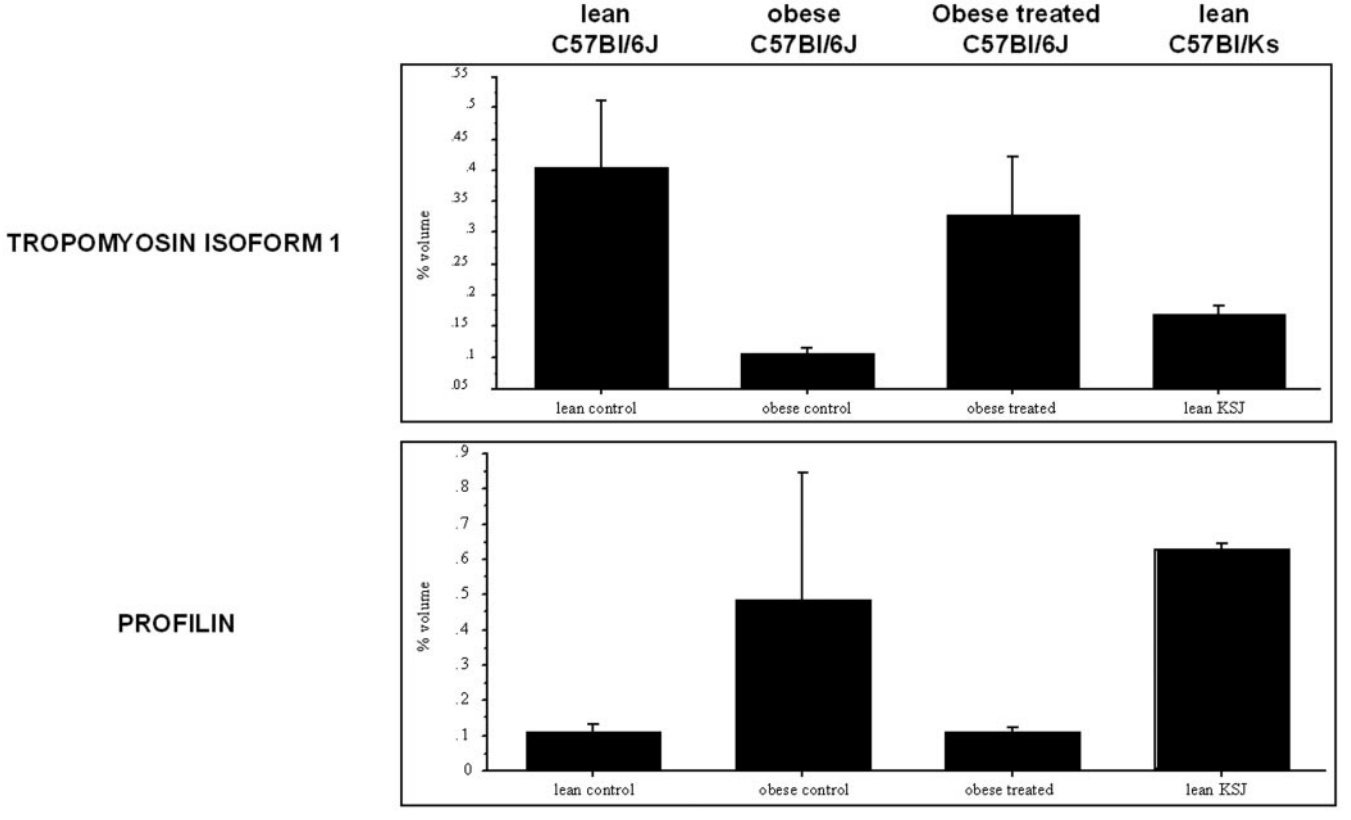

FIG. 6. **Bar charts representing the relative spot volume detected by a differential expression analysis.** Expression of tropomyosin and profilin in islets from lean ($n = 4$), obese ($n = 4$), and obese treated C57Bl/6J ($n = 4$), as well as from lean C57Bl/Ks ($n = 4$), is shown. Expression in both obese mice and lean C57Bl/Ks mice was significantly different from expression in C57Bl/6J mice ($p < 0.05$).

fects actin polymerization (40). Tropomyosin isoform 1 was underexpressed 4-fold, whereas profilin (and a fragment) were overexpressed 4-fold in *lep/lep* mice relative to lean mice, and the expression pattern was normalized by rosiglitazone treatment. Interestingly, C57Bl/KsJ mice showed similar changes in differential expression to the *lep/lep* mice

suggesting that these changes in expression may be important to normal islet cell function. Changes in the actin cytoskeleton network occur during insulin granule secretion, and these changes are regulated by actin-binding proteins. However, the precise roles of tropomyosin and profilin in this process are not clear and neither is the mechanism of how rosiglitazone modulates the expression level.

A common feature of type 2 diabetes is an increased rate of secretion of proinsulin relative to insulin. Thus, in normal subjects proinsulin accounts for 2 to 4% of the total amount of secreted insulin, and it can reach up to 15% in type 2 diabetics (41). Recently it has been shown that, as well as producing evidence of islet cell protection, rosiglitazone treatment of type 2 diabetics reduces the proinsulin output (42). The processing of proinsulin has normally been associated with the activity of carboxypeptidase E (EC 3.4.17.10) (43, 44). This view has been challenged by the finding that mice with the *fat* mutation, a point mutation resulting in S202P and the production of an inactive enzyme that is not secreted, are still capable of producing insulin, albeit at lower levels (45). Fricker *et al.* (46) have provided evidence that carboxypeptidases are present in the secretory pathway and contribute to peptide processing. Carboxypeptidase B has the same enzymatic properties as carboxypeptidase E, but it is normally thought to be associated with exocrine secretion. However, the carboxypeptidase B precursor sequence has been found in an adult mouse islet of Langerhans library. This is consistent with the current finding that carboxypeptidase B precursor protein is found in mouse islets. The 5-fold increase in expression induced by rosiglitazone treatment of both normal and *lep*/*lep* mice suggests that rosiglitazone may have a primary action to increase proinsulin to insulin conversion through an increase in expression of carboxypeptidase B precursor protein.

In conclusion, the array of mouse pancreatic islets by two-dimensional gel electrophoresis and further identification of differentially expressed proteins by mass spectrometric analysis provided evidence that modulation of actin-binding protein expression by BRL49653 may be an important aspect of its protective effect on islet cell structure and function. In addition, rosiglitazone increased carboxypeptidase B precursor protein expression in both *lep*/*lep* and normal mice suggesting that this might be an independent effect of rosiglitazone that contributes to improved insulin processing.

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§ To whom correspondence should be addressed: Geneva Proteomics Center, Central Clinical Chemistry Laboratory, Geneva University Hospital, 24 rue Micheli-du-Crest, 1211 Geneva 14, Switzerland. Tel.: 41-22-372-73-74; Fax: 41-22-372-73-99; E-mail: sanchez@dim.hcuge.ch.

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