Systemic Treatment With Sympatholytic Dopamine Agonists Improves Aberrant β-Cell Hyperplasia and GLUT2, Glucokinase, and Insulin Immunoreactive Levels in *ob/ob* Mice

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Sympatholytic dopamine agonist treatment utilizing bromocriptine and SKF38393 (BC/SKF) significantly lowers basal plasma insulin levels and normalizes basal and glucose-induced insulin secretion of the pancreatic β cell in *ob/ob* mice. While BC/SKF has no significant effect on pancreatic islet cells directly, drug action is mediated via alterations in the hypothalamic-neuroendocrine axis, which drives metabolic changes in peripheral tissues leading to a marked reduction in hyperglycemia and hyperlipidemia and corrects autonomic control of islet function. To elucidate the nature of the functional response of islets to systemic BC/SKF treatment in *ob/ob* mice, we investigated the relative changes in the levels of functionally important β-cell proteins in situ, as well as differences in the β-cell turnover rate, following a 2-week drug treatment. Isolated islets from treated mice exhibit a 3.5-fold increase in insulin content ($P < .01$) that correlated with a 51% reduction in basal plasma insulin levels ($P < .01$) compared with vehicle-treated controls. Using quantitative immunofluorescence microscopy on pancreatic tissue sections, insulin and GLUT2 immunoreactivity of islet β cells of BC/SKF-treated mice were significantly increased (≈ 2.3-fold and ≈ 4.4-fold, respectively; $P < .002$) to the levels observed in islets of their lean littermates. Glucokinase (GK) immunoreactivity was greatly (75%) reduced in β cells from *ob/ob* versus lean mice ($P < .0001$). A modest increase in GK immunoreactivity in β cells of drug-treated mice was observed (≈ 1.6-fold; $P < .05$). Isolated islets from BC/SKF-treated mice exhibit a 42% reduction in DNA content compared with vehicle-treated controls ($P < .01$) to levels observed in lean mice, but without notable differences in islet size. In situ assays for mitosis and apoptosis, using 5-bromodeoxyuridine (BrdU) and terminal deoxyribotransferase (TdT)-UTP nick end labeling (TUNEL) staining techniques, respectively, were performed in pancreas of these mice to determine if β cells show a reduction in hyperplasia following BC/SKF treatment. Accordingly, a pronounced decrease in replicating, BrdU-positive β cells in the drug-treated mice compared with the control group was observed, but without differences in their TUNEL-staining patterns. Collectively, these data suggest that systemic sympatholytic dopaminergic therapy that attenuates hyperglycemia and hyperlipidemia improves islet function in *ob/ob* mice by improving aberrations in the β cell's glucose-sensing apparatus, enhancing insulin storage and/or retention, and stabilizing hyperplasia, thus reducing basal insulin levels.

ISLET β-CELL dysfunction associated with the obese, insulin-resistant state is characterized by an increased basal insulin secretory rate and a blunted glucose-stimulated insulin secretory response.[1-3] The increased basal secretory rate is, in part, due to insulin resistance-induced β-cell compensatory hyperplasia and hypertrophy.[4,5] Also, increased β-cell hexokinase activity and cyclic adenosine monophosphate (cAMP) content may contribute to this enhanced basal secretory activity of islets from obese, insulin-resistant animals.[1,4,6] In a variety of obese, insulin-resistant or diabetic animal models, the attenuation of glucose-stimulated insulin release is coupled to decreases in β-cell levels of insulin and the glucose transporter, GLUT2.[5,7-9] Furthermore, studies of animals with mutant or knock-out genes for β-cell glucokinase (GK), an integral modulator of glucose-stimulated insulin release,[10] and/or GLUT2 indicate that low levels of either may abrogate the normal insulin secretory response to glucose.[10-14]

Evidence from several laboratories suggests that increased levels of circulating free fatty acids (FFA) and glucose may induce these classic features of β-cell dysfunction, namely hyperplasia and decreased GK, GLUT2, and insulin levels.[1,3-5,10,15-16] The mechanism of this lipotoxicity and glucotoxicity to the β cell is complex, but appears to involve the regulation of several transcripiton factors and anaplerotic pathways modulating insulin secretion.[9,17-19]

We have previously demonstrated that systemic treatment of *ob/ob* and *db/db* mice with the sympatholytic dopamine D1 plus D2 agonists, SKF38393 (SKF) plus bromocriptine (BC), respectively, normalizes basal insulin secretory rate and glucose-stimulated insulin release from isolated islets.[1,20] Such systemic treatment also markedly attenuates or normalizes the hyperglycemia and increased plasma FFA levels in these animal models.[21,22] Furthermore, the metabolic responses to dopamine agonist treatment may be elicited by intracerebroventricular administration,[23] and the islet effects cannot be manifested by direct application of agonists to isolated islet preparations.[20] Several lines of evidence indicate that the sympatholytic dopamine agonist effect to normalize islet dysfunction is via the hypothalamic-neuroendocrine-metabolic axis.[24-28]

The mechanism by which BC/SKF treatment improves islet function in the obese diabetic condition may involve influences on β-cell hyperplasia, as well as GLUT2, insulin, and GK levels. However, the effect of such treatment on the above-described cellular biochemical hallmarks of islet dysfunction has never been investigated. Therefore, this study examined the possible influence of systemic SKF/BC treatment of *ob/ob* mice on islet β-cell proliferation status as well as insulin, GK, and

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GLUT2 immunoreactive levels via in situ immunohistochemistry.

MATERIALS AND METHODS

Experimental Design

Five- to six-week-old female C57BL/6J obese (*ob/ob*) and lean (+/?) mice (body weight [BW], 35.7 ± 1.0 and 18.5 ± 0.3, respectively) utilized in this study were maintained from birth on 12-hour daily photoperiods and allowed to feed and drink ad libitum. Mice were divided into 3 groups: (1) lean controls, (2) obese animals receiving daily BC (13 mg/kg/d) and SKF (10 mg/kg/d) injection (intraperitoneal [IP]) at 1 hour after light onset, and (3) vehicle (0.005% ethanol)-injected obese controls. Following 14 days of such treatment, animals were killed at 3 to 4 hours after light onset (ie, at 26 to 27 hours after final injection) to assess islet physiology. Mice were utilized for either (1) islet isolation and plasma studies to determine islet DNA and insulin contents and plasma insulin levels, respectively (n = 7 to 10/group) or (2) islet fluorescence microscopy studies to evaluate islet cell peptide hormone distributions, GLUT2 and GK levels, and mitotic/apoptotic rate (n = 4/lean and 7/each obese group).

Islet Isolation Studies

Determination of plasma insulin and islet DNA and insulin contents. Plasma insulin level was measured by RIA (Linco Research, St. Charles, MO) as previously described[1] from blood isolated via tail vein bleeds prior to sacrifice. Pancreatic islets were isolated from mice by collagenase digestion followed by Ficoll gradient centrifugation.[20] A sample of 20 islets was collected and stored at −80°C until measurements of DNA and insulin content were performed. Islet insulin content was extracted in acid-ethanol[1,20] and quantified by RIA (Linco). The DNA content in islet tissue was determined using a fluorometric method using a DyNA Quant 200 Fluorometer (Hoefer Pharmacia Biotech, San Francisco, CA).[29]

Islet Fluorescence Microscopy Studies

Histology and tissue processing for immunofluorescence. Pancreas and liver were quickly removed from mice euthanized at 4 hours after light onset on the day following their final treatment and immersion-fixed in 4.0% paraformaldehyde in 0.1 mol/L phosphate-buffered saline (PBS, 0.1 mol/L phosphate salts + 144 mmol/L NaCl, pH 7.4) overnight at 4°C. Following washing in several changes of 0.1 mol/L PBS over 2 hours at 4°C, tissues were dehydrated in ascending ethanol concentrations and infiltrated and embedded in Paraplast-X-tra (Fisher Scientific, Pittsburgh, PA). Five-micron sections were sliced and mounted onto charged micro-slides (Charge-Plus, Fisher Scientific). Routine hematoxylin and eosin (H&E) staining was performed on all tissue blocks for histologic evaluation. Micrographs of H&E-stained pancreas sections were recorded on Ektachrome 400 film (Eastman Kodak, Rochester, NY), digitized in a Polaroid Slide Scanner (Polaroid, Cambridge, MA), and formatted and processed on a Power Macintosh computer (Apple Computer, Cupertino, CA) running Adobe Photoshop v. 4.0.

Multiple-labeling immunofluorescence for islet peptide hormones. For qualitative and semiquantitative analyses of islet peptide hormone expression in islet tissue sections, a multiple-labeling protocol was adopted, modified from a previous method.[30] Hydrated sections from each group were simultaneously equilibrated in PBS (10 mmol/L phosphate salts + 144 mmol/L NaCl), then blocked for 1 hour in PBS + 5% normal donkey serum + 1% bovine serum albumin (BSA). Sections from each group were then simultaneously incubated for 12 hours at 4°C in a mixture of primary antisera: guinea pig anti-insulin, 1:2,000, rabbit antiglucagon, 1:1,000 (both from Linco Research), and sheep antisomatostatin, 1:2,000 (Cortex Laboratories, San Leandro, CA) diluted in PBS + 0.1% Triton X-100 + 1% BSA. Following washing in PBS + 0.1% Triton, sections were incubated with a mixture of secondary antibodies: donkey antiguinea pig-amino methyl coumarin (AMCA) (1:500), donkey antirabbit-CY2 (1:250), and donkey antisheep-CY3 (1:2,000) (all from Jackson ImmunoResearch, West Grove, PA). Following washing, the sections were rinsed in distilled water and mounted in Aqua-Polymount (Polysciences, Warrington, PA). All controls for antibody specificity were performed and have been detailed previously.[30]

GK and GLUT2 immunofluorescence. Both GK and GLUT2 immunofluorescent staining were performed simultaneously on 5 μm pancreas sections from all groups of animals as specified previously.[31] The antibodies used were a rabbit antirat C-terminal GK antiserum used at 1:1,000 (gift of M. Tal, Albert Einstein College of Medicine, Bronx, NY) and a rabbit antimouse C-terminal GLUT2 antiserum used at 1:2,000 (gift of B. Thorens, University of Lausanne, Lausanne, Switzerland). Specificity of antisera was confirmed on Western blots of whole cell homogenates of liver (not shown). The secondary antibody consisted of donkey antirabbit-CY3 (1:2,000). Sections were counterstained with the Hoechst 33342 bisbenzamide nuclear marker (0.5 μg/mL, Molecular Probes, Eugene, OR).

Islet mitosis/apoptosis analyses. Tissue 5-bromodeoxyuridine (BrdU)-labeling was used to mark cells in the S phase of the cell cycle (thereby indicating active mitosis). Mice were injected IP with BrdU (Zymed Laboratories, South San Francisco, CA; 50 mg/kg BW) 3 hours before sacrifice. A segment of duodenum was processed in parallel with pancreas and liver to serve as a positive control for both zonal BrdU incorporation and apoptosis (see below). A modified immunolabeling protocol for BrdU incorporation was devised to fluorescently label cycling islet cells. Sections from all groups were simultaneously treated following the manufacturer's directions in the BrdU Staining Kit (Zymed Laboratories), except that strepatavidin-CY3 (1:2,000, Jackson ImmunoResearch) was substituted for avidin-peroxidase in the final labeling step. Sections were counterstained with anti-insulin (using CY2 or AMCA as detailed above) and Hoechst to mark nuclei. Sections of duodenum served as positive controls for BrdU incorporation in the crypt zones.

For determination of islet cell apoptotic activity, a modified TUNEL (terminal deoxyribotransferase {TdT}-UTP nick end labeling) staining protocol was devised to fluorescently mark potentially apoptotic islet cells with fragmented DNA strands. A FragEL-Klenow DNA Fragmentation Kit was used (Oncogene Research Products, Boston, MA) with the initial steps of the protocol followed by the vendor's suggestions. Modification of the kit (Klenow-enzyme based end-labeling with biotin-UTP as the labeled nucleotide) included incubation with strepatavidin-CY3 (1:2,000, Jackson ImmunoResearch) instead of avidin-peroxidase in the final labeling step. Sections were counterstained as detailed above and mounted in Aqua-Polymount. As in the BrdU staining experiments, duodenum served as a positive control, with TUNEL-positive apoptotic cells sited at the tips of villi.

Digital micrography and semiquantitative image analysis. Digital capture of images of multiple-labeled fluorescent-stained sections was conducted using a Nikon Diaphot 300 inverted microscope (Nikon, Japan) with a PlanApo 60x objective lens and a xenon arc lamp with appropriate filter cubes for ultraviolet (UV) (AMCA/Hoechst), fluorescein, and CY3 excitation/emission. The microscope was equipped with an MTI 72S CCD camera coupled to a Ludl filter changer/shutter control system and a framegrabber card interfaced with a Windows-based PC running IP-Lab (Scanalytics, Fairfax, VA) image acquisition software with the Multi-Probe extension for multiple channel overlay analyses.

Pancreatic sections for comparative BrdU, TUNEL, glucagon, somatostatin, insulin, GK, or GLUT2 immunostaining were completed in parallel such that lean, *ob/ob* control, and BC/SKF-treated samples

were processed identically in the same batch of staining reagents. All sections for either insulin, GK, or GLUT2 were imaged on the same day to minimize potential technical variations. A total of 5 to 7 different islets/animal were imaged in the gray-scale mode using IP Lab software with identical camera sensitivity settings for each marker. CY3 was chosen as the optimal fluorophore to indirectly immunomark islet proteins due to its brightness and photostability. For each islet field imaged (area ≈ 220 × 165 μm), the microscope was adjusted to maximize the number of cells with their nucleus in focus (typically more than 95%). Grayscale images (640 × 480 pixel resolution upon digitization) were then analyzed using ScionImage software v. 1.0 (Scanolytics) with the approximate β-cell mass/islet outlined (as determined by insulin counterstaining in GK and GLUT2 experiments) and measured as the mean pixel intensity within the outlined area (in 256 gray scale units). Three nonoverlapping nonislet areas/image were also measured to calculate the average background intensity/microscopic field. Background values were then subtracted from the values obtained from the circumscribed β-cell mass, which was then expressed at the corrected mean pixel intensity/islet section. Values were transferred to Microsoft Excel (Microsoft, Redmond, WA) for statistical analyses, which included calculation of the mean fluorescence intensity/group/islet protein ± SEM.

Digitally merged red-blue-green images were acquired with IP Lab/Multi-Probe on the imaging workstation, then transferred to and processed on a Power Macintosh computer running Adobe Photoshop v. 4.0, and finally formatted using Adobe Illustrator v. 7.0.

Statistics. Significant differences among treatment groups in islet insulin and DNA contents, as well as in immunofluorescent insulin, GK, and GLUT2 levels were determined by Student's *t* test.

RESULTS

Effect of BC/SKF Treatment on Plasma Insulin and Islet DNA and Insulin Content

Relative to lean controls, *ob/ob* mice exhibited a 30-fold increase in plasma insulin level, 62% decrease in islet insulin content, and over a 2-fold increase in islet DNA content ($P < .01$). Following a 14-day treatment with BC/SKF, plasma insulin levels of *ob/ob* mice were reduced by 51% relative to vehicle-treated ob/ob controls (from 45 ± 9 to 22 ± 6 ng/mL; $P < .05$), but still much greater than in lean controls (1.4 ± 0.4 ng/mL). Insulin content of isolated islets from treated *ob/ob* mice was over 3.5-fold greater ($P < .01$) than in the *ob/ob* control group and similar to the level observed in lean mice. DNA content of isolated islets of BC/SKF-treated mice was reduced by 43% ($P < .01$) relative to *ob/ob* controls to levels observed in lean mice (Table 1).

Islet Morphology

Islets from lean and obese control and drug-treated mice were histologically examined in situ to establish potential changes in islet morphology and cytoarchitecture. Islets from *ob/ob* control and BC/SKF-treated mice were generally much larger than those of their lean (+/−) littermates, primarily as a result of β-cell hypertrophy.[32,33] No differences in islet histology were discernible between *ob/ob* control and BC/SKF-treated mice (Fig 1A through C). Drug treatment had no apparent effect on the number or distribution of the major non-β islet cells (Fig 1D and E).

Table 1. Comparison of Islet DNA and Insulin Content From Lean, ob/ob-Vehicle and ob/ob-BC/SKF-Treated Groups

	Day 0	Day 14
DNA content (ng/islet)		
Lean	Not determined	20 ± 3*
ob/ob Vehicle	39 ± 3	43 ± 5
ob/ob BC/SKF	(before grouping)	25 ± 2*
Insulin content (fmol/ng DNA)		
Lean	Not determined	267 ± 59*
ob/ob Vehicle	111 ± 12	100 ± 20
ob/ob BC/SKF	(before grouping)	344 ± 49*

NOTE. Values ± SEM of pooled pancreatic islets from each of 7 to 10 animals per group.

*Denotes a significant difference from the ob/ob vehicle group; $P < .01$.

Islet Hormone Content

Quantitative image analysis of islet glucagon and somatostatin immunoreactivity at the level of individual α and δ cells, respectively, showed no significant differences among the 3 groups in the relative levels of these hormones (results not shown). However, notable increases in insulin immunoreactivity were observed among islets from BC/SKF-treated *ob/ob* mice relative to *ob/ob* controls (Fig 1E and F). Correspondingly, quantitative image analysis of insulin immunoreactivity in islet sections showed a significant 2.3-fold increase of insulin immunoreactivity in islets of drug-treated versus control *ob/ob* mice ($P < .002$) to levels observed in lean mice thus corroborating the results from the RIA insulin content measurements from isolated islets (Table 1).

GLUT2 Expression

GLUT2 immunofluorescence analysis of islet sections showed low levels of cytoplasmic signal in β cells from *ob/ob* versus lean mice (80% decrease; $P < .0001$), as established in a variety of diabetic animal models.[7-9] Surface-oriented GLUT2 was not detected in β cells in the untreated ob/ob mice. However, an increase was observed in β-cell GLUT2 immunoreactivity in the drug-treated *ob/ob* group (≈ 5-fold relative to ob/ob control, $P < .0002$) (Fig 2D through F), approaching the mean value of the lean mouse islets (Fig 2C). No differences in GLUT2 staining intensity within hepatocytes of liver sections were observed between any groups (not shown).

GK Expression

Comparative immunofluorescent staining of GK in pancreas sections showed low to undetectable levels in islets of vehicle-treated *ob/ob* mice versus much (5-fold) higher levels in lean mice ($P < .0001$). However, an enhancement in GK immunoreactivity among BC/SKF-treated *ob/ob* mice relative to *ob/ob* controls was observed. Islet GK immunoreactivity was increased by 50% in *ob/ob*-treated versus *ob/ob* control mice ($P < .05$).

Cell Proliferation and Apoptosis in ob/ob Islets

To determine the cellular processes accounting for the dramatic BC/SKF-induced reduction (normalization) in islet DNA

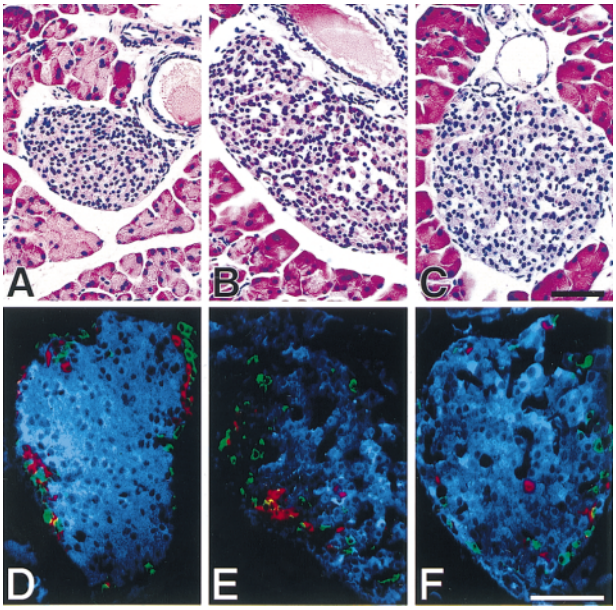

Fig 1. Histologic and immunohistochemical comparison of islets from lean (*ob*/?) (A and D), *ob/ob* control (B and E), and BC/SKF-treated (C and F) mice. (A through C) Hematoxylin and eosin-stained. Although islets from *ob/ob* mice are generally much larger than those of lean littermates, no notable histologic differences were found between control and drug-treated *ob/ob* islets. (D through F) Multiple-labeling immunofluorescence for insulin (blue), glucagon (green), and somatostatin (red). Although there are striking differences in insulin staining between islets from *ob/ob* control and treated groups, no detectable differences were found in the number of α or δ cells or in the relative intensity of immunostaining for glucagon and somatostatin, respectively. BC/SKF treatment normalized insulin immunoreactivity in *ob/ob* islets. Scale bars represent 50 μm.

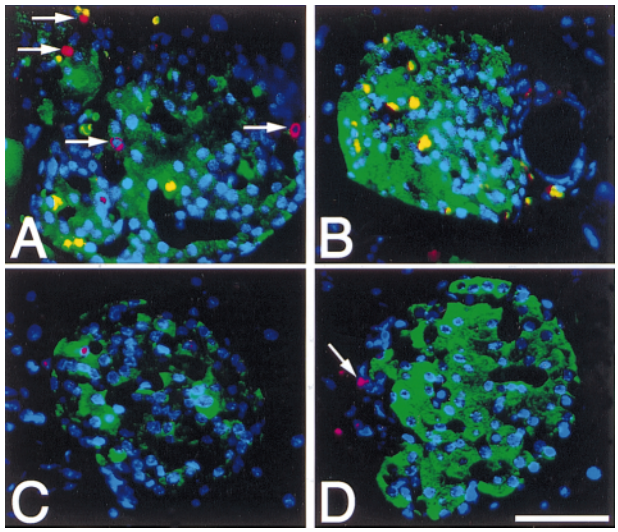

Fig 3. In situ cell proliferation and apoptosis assays in *ob/ob* islets. (A and C) *ob/ob* control. (B and D) BC/SKF-treated. (A and B) Representative BrdU immunolabeling (red) with insulin (green) and Hoechst nuclear counterstaining (blue). (A) Islets from *ob/ob* control mice characteristically display β cells with a high proliferation rate as evidenced by BrdU-positivity (arrows). (B) Islets from BC/SKF-treated mice exhibit islets with very few or no BrdU-positive β cells, similar to those from lean control animals (not shown). (C and D) TUNEL labeling for fragmented DNA (red), suggestive of apoptotic cells, shows no differences in labeling patterns in islets from control and drug-treated groups. TUNEL-positive β cells were extremely rare in all groups, and the few positive islet cells encountered were non-β cells (arrow). Insulin (green) and Hoechst nuclear counterstaining (blue). Scale bars represent 50 μm.

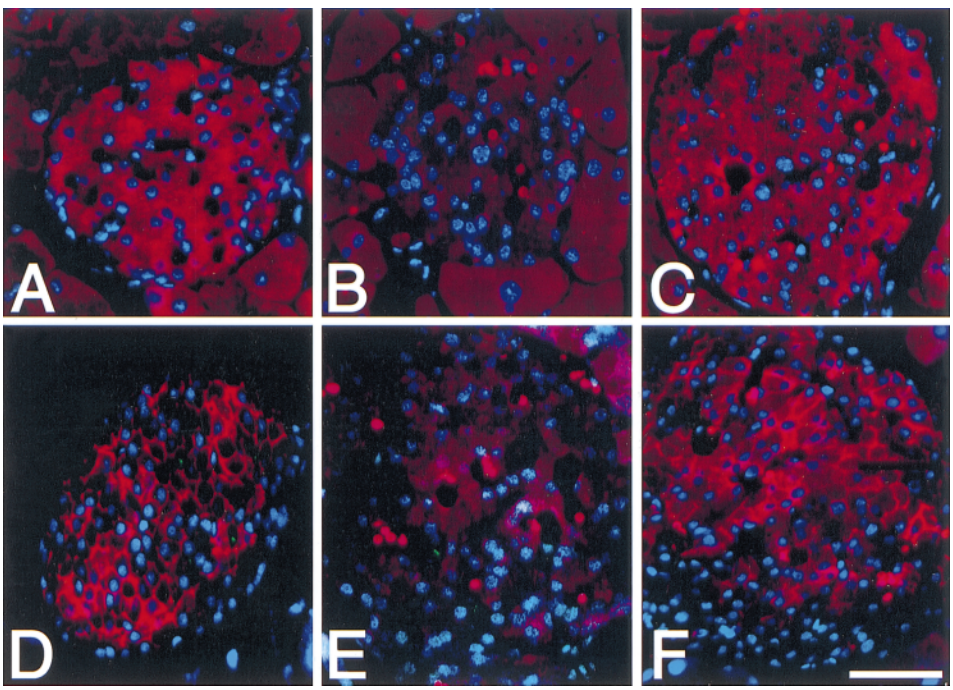

Fig 2. GK and GLUT2 immunofluorescence in islets from lean (*ob*/?) (A and D) *ob/ob* control (B and E), and BC/SKF-treated (C and F) mice. (A through C) Representative GK immunostaining (red) with Hoechst nuclear counterstaining (blue). Drug-treated *ob/ob* mice exhibit islets with enhanced GK immunoreactivity compared with the vehicle-treated control mice. (D through F) GLUT2 immunostaining (red) with Hoechst nuclear counterstaining (blue). Similarly, islet GLUT2 levels become significantly restored following 14 days BC/SKF treatment. No differences in hepatocyte GLUT2 immunoreactivity were observed among the mouse groups. Scale bars represent 50 μm.

content of *ob/ob* mice, we performed in situ cell proliferation assays using BrdU incorporation and subsequent immunolabeling, as well as TUNEL assays for marking probable apoptotic cells in pancreas sections. Not surprisingly, most islets examined from *ob/ob* control mice exhibited several BrdU-positive β-cell nuclei after a 2-hour pulse, indicating mitotically-active cells (Fig 3A). The number of mitotically-active cells was roughly proportional to the size of the islet. In contrast, islets from BC/SKF-treated *ob/ob* mice routinely displayed very few or no BrdU-positive nuclei in islet cells (Fig 3B) similar to the BrdU labeling pattern observed in islets from lean mice (data not shown). This BC/SKF effect was demonstrable after only 7 days of treatment (not shown) suggesting that cellular proliferation had been modified within 1 week of treatment.

The TUNEL labeling patterns in islets of 7- (not shown) or 14-day–treated *ob/ob* mice were not different from that of respective *ob/ob* controls (Fig 3C and D). In fact, double-labeling immunofluorescence showed that TUNEL-positive β cells were rare in islets from all animal groups (lean, *ob/ob*, and *ob/ob*–treated) in these studies.

Mean levels of islet insulin, GK, and GLUT2 immunoreactivity for all groups of mice, as determined by semiquantitative image analysis, are depicted in Fig 4.

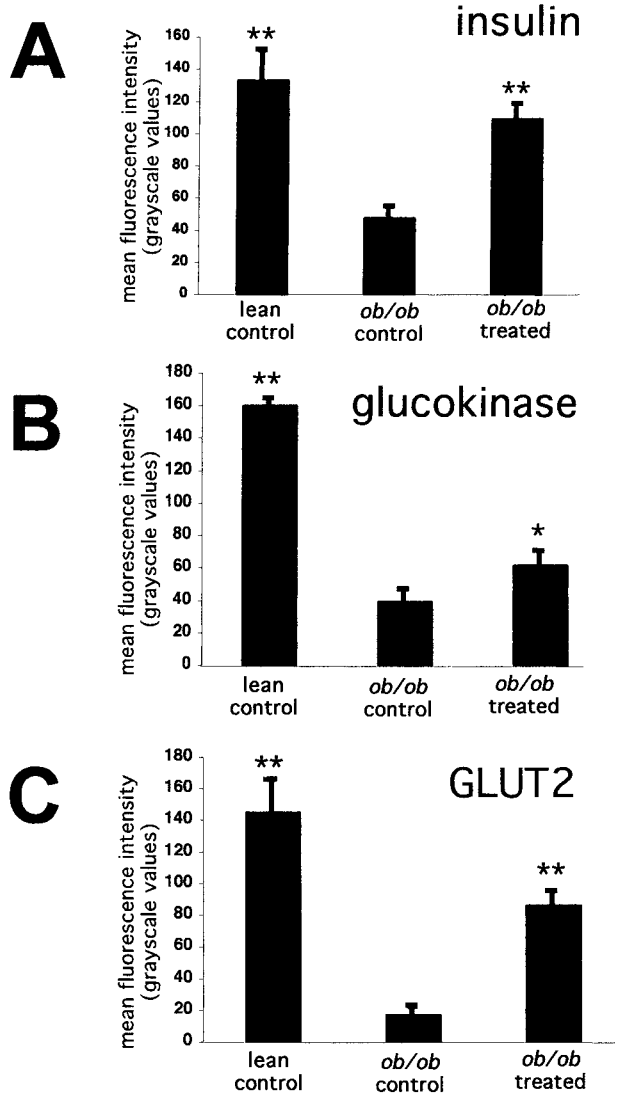

Fig 4. Comparison of islet protein immunoreactivity in situ from lean, *ob/ob* control, and BC/SKF-treated mice as determined by semiquantitative image analysis. Histologic sections of pancreas including 5 to 7 islets/animal were immunofluorescently stained for insulin (A), GK (B), and GLUT2 (C) and subsequently digitally analyzed. Values represent the mean corrected fluorescence intensity ± SEM of 5 to 7 pancreatic islet sections from each of 7 animals/each *ob/ob* group and from each of 4 animals/lean group. (See Materials and Methods section for details). Asterisks denote significant differences from vehicle-treated *ob/ob* controls; *$P < .05$, **$P < .001$.

DISCUSSION

We previously established that BC/SKF treatment of *ob/ob* mice reversed the abnormally increased basal and decreased glucose-stimulated insulin release from isolated islets.[1] This study investigated influences of BC/SKF treatment of *ob/ob* mice on aspects of islet physiology known to participate in the regulation of β-cell insulin secretion. BC/SKF treatment of *ob/ob* mice reversed the decreased expression of β-cell GLUT2 protein and insulin to levels approaching those observed in lean mice. Furthermore, such treatment markedly attenuated the hyperproliferative activity of islet β cells to levels typical of lean mice. Interestingly, this BC/SKF treatment does not alter extractable GK activity,[1] but does marginally increase GK immunoreactivity towards that found in lean mice. Collectively, these islet biochemical and physiologic responses to BC/SKF treatment may, in part, explain the BC/SKF effect to normalize islet insulin secretion in these mice.

The mechanism by which BC/SKF treatment induces the herein described corrections in classic β-cell pathology associated with the obese diabetic state is not elucidated by the present study and remains to be fully delineated. However, the existing fund of knowledge regarding BC/SKF effects on metabolism implicates 2 possible scenarios for such a dopamine agonist influence as described herein via (1) reductions in circulating FFA and glucose levels and (2) normalization of hypothalamic activities regulating islet function. A short review of BC/SKF influences on metabolism may provide a better understanding of its actions on β-cell pathology of type 2 diabetes as follows.

First, direct administration of BC/SKF to isolated islets does not produce the effect of its systemic administration in vivo to normalize β-cell insulin secretory activity in *ob/ob* or *db/db* mice.[1,20] Second, the hypothalamus is a main target of sympatholytic, dopamine agonist action to improve peripheral metabolic, autonomic, and neuroendocrine functions in obese type 2 diabetic models including *ob/ob* mice[23-28] that, in turn, may regulate β-cell function. For instance, sympatholytic dopamine agonist administration normalizes both the increased ventromedial hypothalamic (VMH) release of norepinephrine and the VMH postsynaptic hypersensitivity to norepinephrine in obese type 2 diabetic models.[23,25] Increased VMH noradrenergic drive induces the obese, glucose intolerant, hyperinsulinemic. hyperlipidemic (including elevated plasma FFA level) state, as well as a loss of normal β-cell function.[26,27,34] Furthermore, we

have recently demonstrated that increased peripheral norepinephrine levels, which can be induced by VMH noradrenergic stimulation,[26,35] actually increase insulin release in *ob/ob* mice, likely via stimulation of glucagon release and increased islet insulin secretory responsiveness to glucagon.[36] This peripheral norepinephrine effect is blocked by BC/SKF.[36] In essence, increased VMH norepinephrine activity in vivo desensitizes islets to the direct inhibitory effects of peripheral norepinephrine on insulin secretion.[27] Also, VMH norepinephrine stimulates increased insulin secretion via activation of vagal afferents to the islets.[37,38] Moreover, BC/SKF treatment substantially reduces elevated hypothalamic neuropeptide Y (NPY) levels and paraventricular-dorsomedial tract coticotropin-releasing hormone immunoreactivity in ob/ob mice.[28] This action, in effect, will reduce abnormal elevations in parasympathetic stimulation of insulin secretion and sympathetic stimulation of hepatic glucose output (and hyperglycemia) and adipose lipolysis (and hyperalipacidemia), respectively.[29,39,40] Consequently, BC/SKF-induced normalization of islet hypersecretion of insulin and glucose-stimulated insulin secretory dysfunction of *ob/ob* mice may be a function of its hypothalamic impact to normalize hyperglycemia, elevated plasma FFA levels, and autonomic control of insulin secretion.[1,20-28] Such BC/SKF treatment also reduces islet triglyceride level,[1] which may induce islet insulin secretory dysfunction.[5,18]

Islet β-cell hyperplasia is, in part, an adaptive response to insulin resistance and the excess fuel loads of hyperglycemia and elevated ambient FFA levels[4,5,10] that are all corrected by BC/SKF administration. The effect of BC/SKF to normalize hyperplasia in *ob/ob* β cells appears to be a function of reducing proliferation activity to rates observed in lean mice while minimally influencing apoptosis. However, because apoptotic β cells may be rapidly engulfed by nearby phagocytes, the possibility exists that detection of TUNEL-positive β cells in this study may have been hindered by an acelerated rate of phagocytosis. Similarly, the decline of islet insulin levels in *ob/ob* mice may be, in part, a function of increased fuel load. For example, fatty acids inhibit glucose-induced proinsulin biosynthesis in β cells[4] while enhancing basal insulin release.[5,41] In the type 2 diabetic β cell, insulin secretory rate exceeds the biosynthetic rate leading to a progressive depletion in insulin stores.[5] Therefore, BC/SKF-induced reduction of fuel load to the β cell, as described above, may account for its effect to normalize β-cell hyperplasia and reduce insulin level. A further study in these mice comparing the islet effects of BC/SKF versus those of another antihyperglycemic, antihyperlipidemic agent may be useful in helping to delineate those islet responses to BC/SKF that are specific to BC/SKF compared with those associated with reducing hyperglycemia and hyperlipidemia per se.

The BC/SKF-induced improvement of β-cell GLUT2 level is more complicated. Although islet GLUT2 levels are reduced in vivo in hyperglycemic-hyperlipidemic states,[5,7-9] neither normalization of hyperglycemia nor induction of hyperlipacidemia affect GLUT2 levels (reviewed in Unger,[5] Orci et al,[42] and Chen et al[43]). It has been suggested[18] and we have demonstrated[1] that elevated glucose plus FFA levels synergize to induce islet glucose sensing dysfunction (termed glucolipoxia[18]) and such a circumstance may apply to GLUT2 regulation as well. Therefore, conceivably, the simultaneous normalization of hyperglycemia and hyperlipacidemia via BC/SKF may, in part, also potentiate normalization of islet GLUT2 levels. Although the reduction of β-cell GLUT2 levels, characteristic of type 2 diabetes, may not necessarily be a primary factor contributing to impaired insulin secretion,[44,45] its reduced expression is a marker for β-cell transformation to the diabetic state.[7-9,17,46] Also, GLUT2 may be required for glucose responsiveness via mechanisms other than glucose transport (reviewed in Unger[5]). In several models of diabetes, β-cell loss of GLUT2 transporter protein precedes the loss of immunoreactive insulin,[8,46] and recent evidence suggests that the 2 proteins may be regulated by similar transcription factors, IDX-1 (IPF-1, STF-1, PDX-1).[9,47] Inasmuch as reduced GLUT2 and insulin islet levels of *ob/ob* mice were restored to near normal or normal with BC/SKF treatment, it may be possible that this neuroendocrine modulator may regulate IDX-1 and/or other transcription factors, and further studies along these lines are warranted. For instance, it has been shown that pdx-1, encoding the homeoprotein PDX-1 (IDX-1 in rat), is repressed by fatty acid,[48] elevated plasma levels of which, in turn, are normalized by BC/SKF.[21,22]

An increased fuel load on the β cell may also influence GK activity.[10] Increased ambient FFA levels inhibit β-cell GK activity.[16] In this regard, the GK findings of this study are curious and worthy of specific discussion. First of all, we have previously demonstrated that extractable GK activity is increased in *ob/ob* versus lean mice,[1] and several other studies have likewise demonstrated increased GK activity in other obese type 2 diabetic models.[49-52] However, islet GK immunohistochemical reactivity was markedly reduced in *ob/ob* compared with lean mice (present study). This seeming paradox may well be explained by the finding that GK is reported to translocate from a localized perinuclear compartment to a more diffuse cytoplasmic distribution in response to high ambient glucose levels.[53] Such glucose-activated translocation may be associated with the release of GK from an intracellular binding site of protein character that confers inhibition of GK activity.[54] The greater fraction of cellular GK in *ob/ob* mice may be in the inhibited bound form that is increased in absolute level relative to lean mice due to chronic hyperlipacidemia and hyperglycemia[5,10,48]and thus undetected by immunohistochemistry utilizing an antibody to the C-terminal region of GK. Therefore, the reduced immunoreactive levels of GK protein observed in *ob/ob* mice may reflect the reduced free cytoplasmic active fraction,[54] however, GK activity following its islet extraction, which may release GK from its inhibitory protein modulator, is increased in *ob/ob* mice[1] and other obese type 2 diabetic models.[49-52] In this scenario, the BC/SKF-induced normalization of hyperglycemia and hyperlipacidemia associated with an increase in GK immunoreactivity may represent an increase in the free active form of the enzyme. BC/SKF treatment fully restores the loss in glucose-stimulated insulin release in *ob/ob* mice,[1] a function tightly correlated with GK activity.[55] Although our immunohistochemistry analysis of GK was not sufficiently sensitive to determine subcellular distribution differences in immunoreactive protein, only a moderate increase was observed in GK protein immunoreactivity of *ob/ob* mice following BC/SKF treatment. The lack of complete

normalization of GK enzymatic activity and immunoreactive protein level coupled to a restoration of glucose-stimulated insulin secretion in ob/ob mice following BC/SKF treatment is intriguing and may somehow relate to the lack of leptin in these mice, a known modulator of islet insulin secretion.[56,57] It is also conceivable that a longer treatment with BC/SKF may have produced greater effects on GK immunoreactivity.

Previous studies have demonstrated that BC/SKF treatment normalizes basal and glucose-stimulated insulin secretion from isolated islets of ob/ob and db/db mice.[1,20] This study indicates that such BC/SKF effects may be mediated by its influences on islet β-cell hyperplasia and GLUT2, insulin, and GK contents. Available evidence suggests that BC/SKF effects to normalize hyperglycemia and elevated plasma FFA levels in ob/ob mice may support these observed BC/SKF effects on islet β-cell pathology. Further research of these central sympatholytic dopamine agonist activities may lead to novel approaches for the treatment of type 2 diabetes.

REFERENCES

1. Liang Y, Lubkin M, Sheng H, et al: Dopamine agonist treatment ameliorates hyperglycemia, hyperlipidemia, and the elevated basal insulin release from islets of ob/ob mice. Biochim Biophys Acta 1405:1-13, 1998
2. Leahy JL: Impaired β-cell function with chronic hyperglycemia: "Overworked β cell" hypothesis. Diabetes Rev 4:298-319, 1996
3. Lee Y, Hirose H, Ohneda M, et al: β-Cell lipotoxicity in the pathogenesis of non-insulin-dependent diabetes mellitus of obese rats: Impairment in adipocyte- β-cell relationships. Proc Natl Acad Sci USA 91:10878-10882, 1994
4. Milburn J, Hirose H, Lee Y, et al: Pancreatic beta-cells in obesity. Evidence for induction of functional, morphologic, and metabolic abnormalities by increased long chain fatty acids. J Biol Chem 270: 1295-1299, 1995
5. Unger RH: Lipotoxicity in the pathogenesis of obesity-dependent NIDDM. Diabetes 44:863-870, 1995
6. Cockburn BN, Ostrega DM, Sturis J, et al: Changes in pancreatic islet glucokinase and hexokinase activities with increasing age, obesity, and the onset of diabetes. Diabetes 46:1434-1439, 1997
7. Thorens B, Wu YJ, Leahy JL, et al: The loss of GLUT2 expression by glucose-unresponsive beta cells of db/db mice is reversible and is induced by the diabetic environment. J Clin Invest 90:77-85, 1992
8. Jorns A, Tiedge M, Sickel E, et al: Loss of GLUT2 glucose transporter expression in pancreatic beta cells from diabetic Chinese hamsters. Virchows Arch 428:177-185, 1996
9. Zangen DH, Bonner-Weir S, Lee CH et al: Reduced insulin, GLUT2, and IDX-1 in beta cells after partial pancreatectomy. Diabetes 46:258-264, 1997
10. Matschinsky FM: A lesson in metabolic regulation inspired by the glucokinase glucose sensor paradigm. Diabetes 45:223-241, 1996
11. Guillam M, Hummler E, Schaerer E, et al: Early diabetes and abnormal postnatal pancreatic islet development in mice lacking Glut-2. Nat Genet 17:327-330, 1997
12. Grupe A, Hultgren B, Ryan A, et al: Transgenic knockouts reveal a critical requirement for pancreatic beta cell glucokinase in maintaining glucose homeostasis. Cell 83:69-78, 1995
13. Terauchi Y, Sakura H, Yasuda K, et al: Pancreatic beta-cell-specific targeted disruption of glucokinase gene. Diabetes mellitus due to defective insulin secretion to glucose. J Biol Chem 270:30253-30256, 1995
14. Sakura H, Ashcroft SJ, Terauchi Y, et al: Glucose modulation of ATP-sensitive K-currents in wild-type, homozygous and heterozygous glucokinase knock-out mice. Diabetologia 41:654-659, 1998
15. Zhou Y-P, Grill VE: Long-term exposure of rat pancreatic islets to fatty acids inhibits glucose-induced insulin secretion and biosynthesis through a glucose fatty acid cycle. J Clin Invest 93:870-876, 1994
16. Capito K, Hansen SE, Hedeskov CJ, et al: Fat-induced changes in mouse pancreatic islet insulin secretion, insulin biosynthesis and glucose metabolism. Acta Diabetol 28:193-198, 1992
17. Bonny C, Roduit R, Gremlich S, et al: The loss of GLUT2 expression in pancreatic beta-cells of diabetic db/db mice is associated with an impaired DNA-binding activity of islet-specific trans-acting factors. Mol Cell Endocrinol 135:59-65, 1997
18. Brun T, Roche E, Assimacopoulos-Jeannet F, et al: Evidence for an anaplerotic/malonyl-CoA pathway in pancreatic β-cell nutrient signaling. Diabetes 45:190-198, 1996
19. Farfari S, Schulz V, Corkey B, et al: Glucose-regulated anaplerosis and cataplerosis in pancreatic β-cells. Diabetes 49:718-726, 2000
20. Liang Y, Jetton T, Lubkin M, et al: Bromocriptine/SKF38393 ameliorates islet dysfunction in the diabetic (db/db) mouse. Cell Mol Life Sci 54:703-711, 1998
21. Scislowski PWD, Tozzo E, Zhang Y, et al: Biochemical mechanisms responsible for the attenuation of diabetic and obese conditions in ob/ob mice treated with dopaminergic agonists. Int J Obes 23:425-431, 1999
22. Zhang Y, Scislowski PWD, Prevelige R, et al: Bromocriptine/SKF38393 treatment ameliorates dyslipidemia in ob/ob mice. Metabolism 48:1033-1040, 1999
23. Luo S, Liang Y, Cincotta AH: Intracerebroventricular administration of bromocriptine ameliorates the insulin-resistant/glucose-intolerant state in hamsters. Neuroendocrinology 69:160-166, 1999
24. Luo S, Meier AH, Cincotta AH: Bromocriptine reduces obesity, glucose intolerance and extracellular monoamine metabolite levels in the ventromedial hypothalamus of Syrian hamsters. Neuroendocrinology 68:1-10, 1998
25. Kraszewski KZ, Cincotta AH: Increased responsiveness of ventromedial hypothalamic neurons to norepinephrine in obese versus lean mice: Relation to the metabolic syndrome Int J Mol Med 5:349-355, 2000
26. Cincotta AH, Luo S, Zhang Y, et al: Chronic infusion of norepinephrine into the VMH of normal rats induces the obese glucose-intolerant state. Am J Physiol 278:R435-R444, 2000
27. Liang Y, Luo S, Cincotta AH: Long-term infusion of norepinephrine plus serotonin into the ventromedial hypothalamus impairs pancreatic islet function Metabolism 48:1287-1289, 1999
28. Bina KG, Cincotta AH: Dopaminergic agonists normalize elevated hypothalamic neuropeptide Y and corticotropin-releasing hormone, body weight gain, and hyperglycemia in ob/ob mice. Neuroendocrinology 71:68-78, 2000
29. Liang Y, Najafi H, Smith RM, et al: Concordant glucose induction of glucokinase, glucose usage, and glucose-stimulated insulin release in pancreatic islets maintained in organ culture. Diabetes 41: 792-806, 1992
30. Jetton TL, Liang Y, Pettepher CC, et al: Analysis of upstream glucokinase promoter activity in transgenic mice and identification of glucokinase in rare neuroendocrine cells in the brain and gut. J Biol Chem 269:3641-3654, 1994
31. Jetton TL, Magnuson MA: Heterogeneous expression of glucokinase among pancreatic β-cells. Proc Natl Acad Sci USA 89:2619-2623, 1992
32. Baetens D, Stefan Y, Ravazzola B, et al: Alteration of islet cell populations in spontaneously diabetic mice. Diabetes 27:1-7, 1978
33. Starich G, Zafirova M, Jablenska R, et al: A morphological and

immunohistochemical investigation of endocrine pancreata from obese ob+/ob+ mice. Acta Histochem 90:93-101, 1991

34. Luo S, Luo J, Cincotta AH: Chronic ventromedial hypothalamic infusion of norepinephrine and serotonin promotes insulin resistance and glucose intolerance. Neuroendocrinology 70:460-465, 1999

35. Shimazu T: Neuronal regulation of hepatic glucose metabolism in mammals. Diabetes Metab Rev 3:185-206, 1986

36. Liang Y, Cincotta AH: Increased responsiveness to the hyperglycemic, hyperglucagonemic, and hyperinsulinemic effects of norepinephrine in ob/ob mice. Int J Obes 25:698-704, 2001

37. De Jong A, Strubbe JH, Steffens AB: Hypothalamic influence on insulin and glucagon release in the rat. Am J Physiol 233:E380-E388, 1977

38. Steffens AB, Damsma G, van der Gugten J, et al: Circulating free fatty acids, insulin and glucose during chemical stimulation of hypothalamus in rats. Am J Physiol 247:E765-E771, 1984

39. Sainsbury A, Rohner-Jeanrenaud F, Cusin I, et al: Chronic central neuropeptide Y infusion in normal rats: Status of the hypothalamo-pituitary-adrenal axis, and vagal mediation of hyperinsulinemia. Diabetologia 40:1269-1277, 1997

40. Bernardis LL, Bellinger LL: The dorsomedial hypothalamic nucleus revisited: 1998 update. Proc Soc Exp Biol Med 218:284-306, 1998

41. Bollheimer C, Skelly R, Chester M, et al: Chronic exposure to free fatty acids reduces pancreatic β-cell insulin content by increasing basal insulin secretion that is not compensated for by a corresponding increase in proinsulin biosynthesis translation. J Clin Invest 101:1094-1101, 1998

42. Orci L, Ravazzola M, Baetens D, et al: Evidence that down-regulation of β-cell glucose transporters in non-insulin-dependent diabetes may be the cause of diabetic hyperglycemia. Proc Natl Acad Sci USA 87:9953-9957, 1990

43. Chen L, Alam T, Johnson JH, et al: Regulation of β-cell glucose transporter gene expression. Proc Natl Acad Sci USA 87:4088-4092, 1990

44. Efrat S, Tal M, Lodish HF: The pancreatic beta-cell glucose sensor. Trends Biochem Sci 19:535-538, 1994

45. Tal M, Wu YJ, Leiser M, et al: HRAS downregulates GLUT2 in β-cells of transgenic mice without afecting glucose homeostasis. Proc Natl Acad Sci USA 89:5744-5748, 1992

46. Reddy S, Young M, Poole CA, et al: Loss of glucose transporter-2 precedes insulin loss in the nonobese diabetic and the low-dose streptozotocin mouse models: A comparative immunohistochemical study by light and confocal microscopy. Gen Comp Endocrinol 111: 9-19, 1998

47. Waeber G, Thompson N, Nicod P, et al: Transcriptional activation of the GLUT2 gene by the IPF-1/STF-1/IDX-1 homeobox factor. Mol Endocrinol 10:1327-1334, 1996

48. Gremlich S, Bonny C, Waeber G, et al: Fatty acids decrease IDX-1 expression in rat pancreatic islets and reduce GLUT2, glucokinase, insulin, and somatostatin levels. J Biol Chem 272:30261-30269, 1997

49. Liang Y, Bonner-Weir S, Wu YJ, et al: In situ glucose uptake and glucokinase activity of pancreatic islets in diabetic and obese rodents. J Clin Invest 93:2473-2481, 1994

50. Nesher R, Gross DJ, Donath MY, et al: Interaction between genetic and dietary factors determines β-cell function in *Psammomys obesus*, an animal model of type 2 diabetes. Diabetes 48:731-737, 1999

51. Man Z-W, Zhu M, Noma Y, et al: Impaired β-cell function and deposition of fat droplets in the pancreas as a consequence of hypertruglyceridemia in OLETF rat, a model of spontaneous NIDDM. Diabetes 46:1718-1724, 1997

52. Chan CB: Beta-cell stimulus—Secretion coupling defects in rodent models of obesity. Can J Physiol Pharmacol 73:1414-1424, 1995

53. Noma Y, Bonner-Weir S, Latimer JB, et al: Translocation of glucokinase in pancreatic β-cells during acute and chronic hyperglycemia. Endocrinology 137:1485-1491, 1996

54. Tiedge M, Steffeck H, Elsner M, et al: Metabolic regulation, activity state, and intracellular binding of glucokinase in insulin-secreting cells. Diabetes 48:514-523, 1999

55. Sweet IR, Li G, Najafi H, et al: Effect of a glucokinase inhibitor on energy production and insulin release in pancreatic islets. Am J Physiol 271:E606-E625, 1996

56. Seufert J, Kieffer TJ, Habener JF: Leptin inhibits insulin gene transcription and reverses hyperinsulinemia in leptin-deficient ob/ob mice. Proc Natl Acad Sci USA 96:674-679, 1999

57. Poitout V, Rouault C, Guerre-Millo M, et al: Does leptin regulate insulin secretion? Diabetes Metab 24:321-326, 1998