

Long-Term Treatment with Novel Glycogen Synthase Kinase-3 Inhibitor Improves Glucose Homeostasis in ob/ob Mice: Molecular Characterization in Liver and Muscle

Oksana Kaidanovich-Beilin and Hagit Eldar-Finkelman

Department of Human Genetics and Molecular Medicine, Sackler School of Medicine, Tel Aviv University, Tel Aviv, Israel

Received May 31, 2005; accepted September 14, 2005

ABSTRACT

Glycogen synthase kinase-3 (GSK-3) is critically involved in insulin signaling, and its selective inhibition may present a new therapy for treatment of insulin resistance and type 2 diabetes. The current studies were designed to examine the impact of long-term in vivo inhibition of GSK-3 and its effects in the specific tissues. ob/ob mice were treated daily with one dose (400 nmol, i.p.) of a selective GSK-3 peptide inhibitor, L803-mts, for 3 weeks. Treatment with L803-mts reduced blood glucose levels, improved glucose tolerance, and prevented elevation of hyperglycemia with age. However, L803-mts did not affect either body weight or food consumption and was not toxic, as judged by histopathology and blood chemistry analyses. Consistent with these results, L803-mts suppressed mRNA levels of hepatic phosphoenolpyruvate carboxykinase (PEPCK) (50%) and increased hepatic glycogen content by 50%. On the other hand, L803-mts did not affect glucose

6-phosphate (G-6-P) phosphatase (G-6-Pase) mRNA levels or its enzymatic activity in the liver. Investigation for possible mechanisms responsible for PEPCK suppression indicated that phosphorylation of cAMP-responsive element transcription factor (CREB) at Ser¹³³ was reduced remarkably by L803-mts, which was also associated with reduced phosphorylation at Ser¹²⁹ and no change in total CREB. This suggested that PEPCK was suppressed by GSK-3 inhibition-mediated inactivation of CREB. In skeletal muscle, treatment with L803-mts led both to up-regulation in GLUT4 expression and to a 20% increase in glycogen content. Our studies show that long-term treatment with GSK-3 inhibitor improves glucose homeostasis in ob/ob mice and demonstrates a novel role of GSK-3 in regulating hepatic CREB activity and expression of muscle GLUT4.

Insulin resistance, commonly defined as the inability of peripheral tissues to respond normally to physiological concentrations of insulin, is considered one of the earliest changes associated with the onset of type 2 diabetes. Characterization and identification of cellular targets contributing to defects in the insulin-signaling cascade is a major challenge in diabetic research, and the pharmacologic manipulation of these cellular targets offers beneficial opportunities for treating this common disease.

Glycogen synthase kinase-3 (GSK-3) is a serine/threonine kinase that recently emerged as a potential drug discovery

target in insulin resistance and type 2 diabetes. The enzyme is a downstream target inhibited by insulin (Welsh and Proud, 1993), and its activity under nonstimulated conditions inhibits key downstream targets of insulin receptor, such as glycogen synthase (Woodgett and Cohen, 1984; Roach, 1991) and insulin receptor (IR) substrate-1 (IRS-1) (Eldar-Finkelman and Krebs, 1997; Liberman and Eldar-Finkelman, 2004). Consequently, elevated GSK-3 activity was found in diabetic tissues, such as fat and muscle (Eldar-Finkelman et al., 1999; Nikoulina et al., 2000), possibly contributing to inhibition of the insulin-signaling cascade in these tissues. Therefore, it was proposed that inhibition of GSK-3 may be a useful approach for improving insulin-resistance conditions (Eldar-Finkelman, 2002). This view was further supported by a number of studies showing that selective GSK-3 inhibitors provoked antidiabetic effects in cells and in animal models (Cline et al., 2002; Nikoulina et al.,

This work was supported by the Israel Science Foundation Grant 755/04 and was performed as partial fulfillment of the requirements for a Ph.D. degree (O.K.-B.) in Sackler School of Medicine.

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.
doi:10.1124/jpet.105.090266.

ABBREVIATIONS: GSK-3, glycogen synthase kinase-3; PEPCK, phosphoenolpyruvate carboxykinase; IR, insulin receptor; CREB, cAMP-responsive element transcription factor; G-6-P, glucose 6-phosphate; GS, glycogen synthase; G-6-Pase, glucose-6-phosphatase; FKHR, Forkhead transcription factor; PKB, protein kinase B; IRS-1/2, insulin receptor substrate1/2; GLUT4, glucose transporter 4; GTT, glucose tolerance test(s); PCR, polymerase chain reaction; PCG-1, peroxisome proliferator-activated receptor- γ coactivator-1; UDPG, uridine 5-diphosphate [¹⁴C]glucose.

2002; Henriksen et al., 2003; Plotkin et al., 2003). However, the impact of long-term *in vivo* inhibition of GSK-3 has not been determined. Moreover, the molecular mechanism affected by inhibition of GSK-3 in the specific tissues remains elusive.

Most of protein kinases inhibitors, including those developed for GSK-3 (Coghlan et al., 2000; Nikoulina et al., 2002), are ATP-competitive inhibitors. However, a major drawback of ATP-competitive inhibitors is their limited specificity; therefore, there is a concern that such inhibitors exert undesired side effects.

We developed a novel substrate competitive peptide inhibitor for GSK-3, L803-mts, that was shown to be specific toward GSK-3 (Plotkin et al., 2003). We showed that L803-mts provoked insulin-like effects in cells and in animal model systems (Plotkin et al., 2003). In the current studies, we examined the impact of a long-term administration of GSK-3 inhibitor in the diabetic model of *ob/ob* mice and studied molecular mechanisms provoked by GSK-3 inhibition in the insulin target tissues. The present studies show that daily injections of L803-mts improve glucose homeostasis in *ob/ob* mice and describe L803-mts-specific effects in liver and muscle.

Materials and Methods

Materials. L803-mts [*N*-myristoyl-GKEAPPAPPQS(p)P] and biotin-conjugated peptide bio-L803-mts were synthesized by Genemed Synthesis Inc. (San Francisco, CA), as described previously (Plotkin et al., 2003), and were dissolved in 0.1% dimethyl sulfoxide buffer solution. GLUT1 and GLUT4 antibodies were from Chemicon International (Temecula, CA); PKB and phospho-PKB (Thr³⁰⁸), GSK-3 β , phospho-GSK-3 β Ser⁹, phospho-GSK-3 α Ser²¹, Forkhead transcription factor (FKHR), and phospho-FKHR Ser²⁵⁶ and CREB antibodies were from Cell Signaling Technology Inc. (Beverly, MA). IR, IRS-2, IRS-1 p85 subunit, and phospho-CREB (Ser¹³³) antibodies were from Upstate Biotechnology (Lake Placid, NY). Antiphosphotyrosine antibody (PY99) was from Santa Cruz Biotechnology (Santa Cruz, CA), and phospho-CREB (Ser¹²⁹, Ser¹³³) was from BioSource International (Camarillo, CA). Radioactive materials were purchased from GE Healthcare, Little Chalfont (Buckinghamshire, UK). All other reagents were purchased from Sigma-Aldrich (Rehovot, Israel).

Animals. Male C57BL/6J-obese (Lep^{ob}/Lep^{ob}) mice (10 weeks of age) were purchased from Harlan (Indianapolis, IN) and were acclimated to the animal research facilities for 7 days. Animals were kept in a temperature-controlled room at 24 \pm 1°C, with 12-h light cycles. Mice were fed standard laboratory rodent chow, and water was offered *ad libitum*. The animals were randomized to the saline- and L803-mts-treated groups, and two sets of independent experiments were performed, each including 24 animals.

The mice were injected *i.p.* once per day with L803-mts (400 nmol, 300 μ l) or saline (300 μ l), administered at 12:00 AM for 21 days. At the end of each week, tail-bleed glucose levels, as well as body weight, were determined under nonfasting conditions by 11:30 AM (thus, 24-h post-last dose on the indicated days and before the next injection). Animals were subjected to glucose-tolerance tests (GTT) after 4 h fasting in the morning. Glucose (1 g/kg) was injected *i.p.*, and blood samples were collected from tail vein at 30, 60, and 120 min. Blood glucose levels were immediately measured by Sugar Accutrend Sensor (Roche Diagnostics, Mannheim, Germany). Food consumption was measured at 11:30 AM at the start and end of a 24-h period (same 24-h period each week) and was divided by the number of mice per cage for an index of estimated 24-h food consumption per mouse. After 3 weeks of treatment, mice were sacrificed (with CO₂), and blood samples were immediately collected from

the heart's aorta in tubes. The tubes were placed in ice and centrifuged at 4°C, and the obtained plasma was immediately stored at -80°C. Plasma insulin was determined using insulin radioimmunoassay kit (INSIK-5; DiaSorin SpA, Saluggia, Italy). Serum chemistry was analyzed by American Medical Laboratories (Herzliya Medical Center, Herzliya, Israel). Histopathology analyses were performed on liver, kidney, heart, and pancreas and showed no inflammation or necrosis (diagnosed by Patho Vet Diagnostic Veterinary Pathology Services, Kiryat Weizmann Rehovot, Israel). Apoptosis in Langerhans islets was analyzed in paraffin-embedded tissue sections using ApopTag *situ* apoptosis detection kit (Chemicon International). We did not detect apoptosis of islet cells in control and L803-mts samples. All animal procedures were conducted in compliance with protocols approved by the Animal Research Committee at Tel-Aviv University (Tel-Aviv, Israel).

Tissue Extracts and Western Blots. Liver and skeletal gastrocnemius muscle were removed from the animals and immediately frozen in liquid nitrogen. Tissues were extracted by homogenization in buffer H [50 mM β -glycerophosphate, pH 7.3, 1 mM EGTA, 1 mM EDTA, 50 mM NaF, 1 mM orthovanadate, leupeptin, aprotinin, and pepstatin A (25 μ g/ml each), and 500 nM microcystine LR 1% Triton X-100], with a Polytron homogenizer followed by centrifugation at 15,000g to remove particulate matter. Supernatants were collected, and protein concentration was determined by Bradford assays. Equal amounts of proteins were subjected to gel electrophoresis followed by immunoblot analysis with the indicated antibodies, according to manufacturer's procedures. In some experiments IRS-1 and IRS-2 were immunoprecipitated from cell extracts with specific antibodies and subjected to gel electrophoresis and immunoblotted with antiphosphotyrosine antibody. The intensity of the bands was quantitated with a Bio Imaging System Densitometer (BIS202D; Tel Aviv, Israel).

Peptide Distribution. Biotin-conjugated peptide bio-L803-mts (400 nmol, 300 μ l) was injected *i.p.* into *ob/ob* mice ($n = 3$), and 120 min postinjection, gastrocnemius skeletal muscle, fat, and liver were removed and fixed in 4% buffered paraformaldehyde. Cryostat tissue sections (10–12 μ m) were incubated with Cy3-conjugated Streptavidin (1:4000; Jackson ImmunoResearch Laboratories Inc., West Grove, PA), and the presence of biotin-labeled L803-mts in the tissues was visualized by fluorescence microscopy. The peptide stability was tested in mouse serum as described previously (Plotkin et al., 2003).

Immunohistochemistry was performed in paraffin-embedded sections (5 μ m) of liver or gastrocnemius skeletal muscle ($n = 3$). Tissues were deparaffinized with xylene, rehydrated with ethanol, and then washed in phosphate-buffered saline. The endogenous peroxidase activity was quenched by incubating the sections in 0.3% hydrogen peroxide for 20 min at room temperature. After blocking the sections with 10% nonimmune goat serum in phosphate-buffered saline for 10 min, deparaffinized paraffin sections were reacted for 2 h at room temperature, with specific antibodies against CREB Ser¹³³, CREB, or GLUT4. Detection was checked by an appropriate biotinylated second antibody with streptavidin-peroxidase conjugate and *S*-(2-aminoethyl)-L-cysteine as substrate (Histostain-SP kit; Zymed Laboratories, South San Francisco, CA). Counterstaining was performed with hematoxylin.

Total RNA Isolation and Quantitative Real-Time PCR. Total RNA was isolated from liver samples using TRIzol reagent (Invitrogen, Carlsbad, CA) and treated with RNase-free DNase I (DNA-free; Ambion, Austin, TX). RNA sample quality and concentrations were determined by UV spectrophotometry (average OD_{260/280}, ratio = 1.6–1.8), and RNA integrity was confirmed by gel. Two micrograms of total RNA was reverse-transcribed using the EZ-First Strand cDNA synthesis kit with oligo(dT) primers (Biological Industries, Tel-Aviv, Israel), using an ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA). Reactions were performed in a 20- μ l volume, including diluted cDNA sample (2 μ l), primers (0.5 μ M), and SYBR Green PCR Master Mix (Applied Bio-

systems). The primer sequences used are as follows: phosphoenolpyruvate carboxykinase (PEPCK) forward, 5'-GGTGTCTTACTGG-GAAGGCATC-3', and reverse, 5'-CAATAATGGGGCACTGGCTG-3'; G-6-Pase forward, 5'-CATGGGCGCAGCAGGTGTACT-3', and reverse, 5'-CAAGGTAGATCCGGGACAGACAG-3'; and tubulin $\beta 5$ forward, 5'-CCT GCT CAT CAG CAA GAT CC-3', and reverse, 5'-TCT CAT CCG TGT TCT CAA CC-3'

Routinely, the reactions were cycled 40 times. Melting curve analysis and agarose gel electrophoresis allowed us to verify the specificity of PCR amplifications. Standard curve assays were performed by measuring mRNA transcript levels obtained with specific primer sets from a control cDNA sample. mRNA transcript levels were normalized against tubulin $\beta 5$ at each sample. No-template control assays were performed for each primer set used.

Reverse Transcription-PCR. Peroxisome proliferator-activated receptor- γ coactivator-1 (PGC-1) cDNA (prepared as described in the previous section) was amplified with PGC-1 primers (0.5 μ M), dNTP, and *Taq*DNA polymerase, and the reactions were cycled 30 to 40 times. PCR products were resolved on a 2% agarose gel and visualized by ethidium bromide staining. PGC-1 primers are as follows: forward, 5'-GGAGCCGTGACCACTGACA-3', and reverse: 5'-TG-GTTTGTGTCATGGTTCTG-3' (expected product size 176 bp).

Glycogen Synthase Activity in Liver and Muscle. Liver and skeletal gastrocnemius muscle was removed from the treated and control animals and frozen immediately. The tissue was homogenized with glycogen synthase (GS) buffer (50 mM Tris, pH 7.8, 100 mM NaF, 10 mM EDTA, 5% glycerol, and protease inhibitors 20 μ g/ml leupeptin, 10 μ g/ml aprotinin, 10 mg/ml pepstatin A, and 1 mM benzamide) and centrifuged at 8000g. Glycogen synthase activity was assayed in the supernatants according to the method of Thomas et al. (1968) and based on the incorporation of UDPG into glycogen. Aliquots of tissue homogenate (15 μ l) were incubated with 15 μ l of reaction mixture [66.6 mM Tris, pH 7.8, 32.5 mM potassium fluoride, 0.8 μ Ci/ μ l [14 C]UDPG (400 μ M), and 13 mg/ml glycogen rabbit liver (Sigma-Aldrich, St. Louis, MO)] for 20 min at 30°C. The reactions were then spotted on ET31 (Whatman) papers, washed with 66% ice-cold ethanol, and counted for radioactivity. Glycogen synthase assays were measured in the presence of 0.1 or 10 mM G-6-P. Activity ratios were calculated as activities measured at low G-6-P/high G-6-P.

Quantification of Glycogen. Tissues (100–200 mg) were homogenized, and one aliquot (100 μ l) of homogenate was mixed with 0.5 ml of glucose oxidase reaction buffer [0.1 M phosphate buffer, pH 6.0, 0.01% *O*-dianizidine (Sigma-Aldrich catalog number D9143-5G), 100 mg of glucose oxidase, 80 mg of amyloglucosidase, and 1 mg of peroxidase] and incubated at 37°C for 30 min followed by the addition of 0.5 ml of ice-cold 6 N H₂SO₄. The absorbance of glucose concentrations was read at 540 nm. Residual glucose was determined in samples untreated with amyloglucosidase. In parallel, rabbit liver glycogen type III (0.2 μ g/ml; Sigma-Aldrich) at different concentrations were treated identically and used as standards. The absorbance of standards was plotted against glycogen concentrations to yield a standard curve from which the relative glycogen concentrations in unknown samples were calculated. After the residual glucose concentrations were subtracted, hepatic glycogen contents in L803-mts- and saline- treated group of mice were compared.

G-6-Pase Activity. G-6-Pase activity was measured by the production of phosphate from glucose 6-phosphate. Liver (100 mg) was homogenized by hand-held homogenizer in 0.25 M sucrose-Hepes buffer (pH 7.4). Homogenates were centrifuged at 3000g. Supernatant (100 μ l) was mixed with 10 μ l of taurocholic acid (Sigma-Aldrich catalog number T0750; 44 mg/ml) and was incubated for 30 min on ice followed by the addition of 290 μ l of sucrose-Hepes buffer. One aliquot (60 μ l) was mixed with reaction buffer (25 μ l of 0.5 M Tris buffer, pH 6.5, 20 μ l of 100 mg/ml bovine serum albumin, 40 μ l of 0.1 M glucose 6-phosphate, and 55 μ l of water) and incubated at 37°C for 0 and 20 min followed by the addition of 600 μ l of ice-cold 3.5% trichloroacetic acid. After centrifugation (at 3000g for 10 min at 4°C),

free phosphate in supernatant was determined by incubation with 300 μ l of phosphate reagent: 5% ammonium molybdate tetrahydrate (Sigma-Aldrich catalog number A-7302) dissolved in 4 N HCl and 1g of Ion (II) sulfate heptahydrate [Merck (Darmstadt, Germany); catalog number 3965.0500] for 10 min at room temperature. The absorbance of each sample was read at 650 nm. Another aliquot was used for protein determination by Bradford analysis.

Statistics. Data were analyzed for statistically significant differences between control and treated animals using Origin 6.0 Professional software and Student's *t* test. For all analyses, *p* < 0.05 was considered statistically significant. Variations are given as mean \pm S.E.M.

Results

Metabolic and Physiological Profiles of ob/ob Mice Treated with L803-mts. ob/ob mice were injected i.p. daily with L803-mts for 3 weeks. We first confirmed the presence of L803-mts in tissues. Biotin-conjugated L803-mts (bio-L803-mts) was injected i.p. into animals and showed a fluorescence signal in liver and muscle tissue sections that were stained with streptavidin-Cy3 (Fig. 1A). A fluorescence signal was also observed in fat tissue (data not shown). This result indicated a favorable tissue distribution of L803-mts. Physiological analyses revealed that L803-mts did not change body weight or food consumption during the 3 weeks of treatment (data not shown). However, nonfasting blood glucose levels were reduced significantly in L803-mts-treated animals compared with control animals (saline-treated; Fig. 1B). Reduction in plasma glucose levels occurred within the first 2 weeks of treatment and, unlike control animals, did not increase with age (Fig. 1B). L803-mts improved glucose tolerance, as judged by GTT performed after 2 (data not shown) or 3 weeks of treatment (Fig. 1C). Blood samples were taken at the end of the experiment for further analyses (at nonfasting conditions). Nonfasting insulin levels were unchanged after 1, 2 (data not shown), or 3 weeks of treatment (see Table 1). These results may be explained by the severe hyperinsulinemia, which is a prominent feature of this animals model (Bailey et al., 1975; Song et al., 2002). Blood chemistry analyses showed no significant changes in hepatic function as judged by determination of transaminase, aspartate transaminase, or alkaline phosphatase concentrations (Table 1). There were also no significant differences in serum concentrations of triglycerides, cholesterol, and albumin (Table 1), although it should be noted, however, that lipid metabolism is significantly different in the ob/ob mouse compared with other model systems or human (Cohen et al., 2003). Histological analysis revealed no signs for damage, such as inflammation or necrosis in liver heart kidney, and pancreatic islets were not damaged by L803-mts. These results are important in view of the adverse effects, including body weight gain or triglyceride elevation provoked by other antidiabetic drugs.

Hepatic Glucose Metabolism in L803-mts-Treated Animals. The results described above demonstrated clearly that long-term treatment with L803-mts improved glucose homeostasis in ob/ob mice. Because liver plays a prominent role in maintaining blood glucose levels through hepatic glucose production (HGP), we determined the expression of rate-limiting enzymes for gluconeogenesis, PEPCK, and G-6-Pase, which are rapidly inhibited at the transcriptional level by insulin or fasting (Hanson and Reshef, 1997). The relative

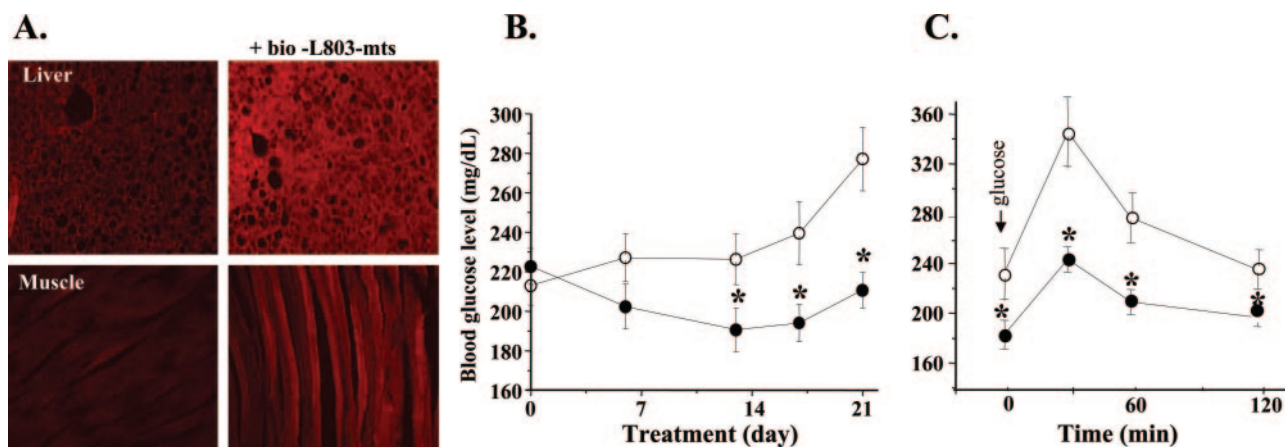


Fig. 1. L803-mts and animal characteristics. A, transduction of the bio-L803-mts peptide inhibitor into liver and muscle tissues. Liver and gastrocnemius muscle tissue section prepared from saline- or bio-L803-mts-injected animals were stained with Cy3-conjugated streptavidin and visualized by fluorescence microscopy (shown initial magnification, 10 \times). B, mean of nonfasting blood-glucose levels determined at indicated days. Filled circles, L803-mts-treated; open circles, control ($n = 19\text{--}22 \pm \text{S.E.M.}$; *, $p < 0.05$ treated versus control). C, GTT experiments performed after 3 weeks of treatment with L803-mts. Filled circles, L803-mts-treated; open circles, control ($n = 12\text{--}14 \pm \text{S.E.M.}$; *, $p < 0.05$ treated versus control). Time = 0; administration of glucose (1 g/kg).

amounts of mRNA levels of PEPCK and G-6-Pase were determined by quantitative real-time PCR. The results presented in Fig. 2A indicated that mRNA levels of PEPCK were reduced significantly by L803-mts (~50%), but mRNA levels of G-6-Pase were comparable between the control and treated samples. We also examined G-6-Pase enzymatic activity, which unlike PEPCK may be regulated at the protein level. G-6-Pase assays were performed in liver extracts, and results shown in Fig. 2B indicated comparable enzymatic activity of G-6-Pase between control and L803-mts-treated animals (Fig. 2B). Thus, *in vivo* inhibition of GSK-3 selectively reduced hepatic PEPCK gene expression.

Hepatic rates of glycogen synthesis are additional determinant that control HGP. Thus, glycogen levels were determined in livers from control and L803-mts-treated animals. Glycogen levels were increased 1.5-fold in the L803-mts-treated livers compared with controls (3.88 ± 0.19 and 5.95 ± 0.12 mg/g tissue of control versus L803-mts treated, respectively, $n = 7$, *, $p < 0.01$). The rate-limiting enzyme for glycogen synthesis is GS, which is also a direct target phosphorylated by GSK-3 (Woodgett and Cohen, 1984; Roach, 1991). GS activity assays were performed in liver extracts in

TABLE 1
Metabolic parameters of control and L803-mts-treated animals
No significant differences were observed between treated and non-treated animals.

| Parameters | Units | Control | L803-mts |
|---|-------|----------------|-----------------|
| Insulin ($n = 8$) | ng/ml | 20.4 ± 1.4 | 22.9 ± 2.6 |
| Hepatic function ($n = 7\text{--}10$) | | | |
| Bilirubin (total) | mg/dl | 0.1 ± 0.05 | 0.03 ± 0.03 |
| Alanine Transaminase | IU/l | 504 ± 200 | 453 ± 146 |
| Aspartate transaminase | IU/l | 653 ± 160 | 770 ± 96 |
| Alkaline phosphatase | IU/l | 244 ± 21 | 225 ± 14 |
| Lipid content ($n = 7\text{--}10$) | | | |
| Cholesterol | mg/dl | 232 ± 9.2 | 218 ± 6.0 |
| High-density lipoprotein cholesterol | mg/dl | 195 ± 6.8 | 185 ± 3.8 |
| Low-density lipoprotein cholesterol | mg/dl | 34.8 ± 2.4 | 32 ± 0.8 |
| Triglycerides | mg/dl | 125 ± 7.8 | 121 ± 6.8 |
| Protein content ($n = 7\text{--}10$) | | | |
| Total protein | g/dl | 6.6 ± 0.25 | 6.5 ± 0.11 |
| Albumin | g/dl | 4.5 ± 0.21 | 4.5 ± 0.08 |

the presence of low or high concentrations of its allosteric activator G-6-P. The activity ratios (i.e., enzyme activity at low G-6-P/enzyme activity at high G-6-P), which represent the active fraction of GS, were similar in control or L803-mts-treated samples (0.019 ± 0.04 and 0.016 ± 0.03 control versus L803-mts treated, respectively, $n = 15$, *, $p < 0.05$), suggesting that inhibition of GSK-3 was not sufficient to activate GS through covalent modification (i.e., phosphorylation regulation). These results may be in agreement with previous works reporting that GS activity is regulated by multiple pathways not necessarily dependent on inactivation of GSK-3 (Fernandez-Novell et al., 1994).

L803-mts Represses CREB Activity in the Liver. Numerous studies indicated a primary role for CREB in hepatic gluconeogenesis and, specifically, the stimulation of PEPCK gene expression (Leahy et al., 1999; Herzig et al., 2001). This prompted us to examine whether CREB activity is influenced by L803-mts. CREB phosphorylation at Ser¹³³ is essential for CREB transcriptional activity, including activation of PEPCK (Parker et al., 1996; Sassone-Corsi, 1998). Indeed, phosphorylation of CREB at Ser¹³³ was sharply reduced in the livers from L803-mts-treated animals (~70% reduction; Fig. 3A); total CREB was unchanged (Fig. 3A). Decreased phosphorylation in Ser¹²⁹, which is a target directly phosphorylated by GSK-3 (Fiol et al., 1994), was observed in L803-mts-treated livers (Fig. 3A), which could add to the inhibition of CREB (Fiol et al., 1994). Immunohistochemistry analysis using specific anti-phospho-CREB (Ser¹³³) antibody further showed a marked reduction in the phosphorylation levels of Ser¹³³ located in the cell nuclei in the L803-mts-treated samples (CREB is predominantly nuclear; Fig. 3B).

In addition to CREB, FKHR or PGC-1 is an important mediator of G-6-Pase and PEPCK gene expression (Nakae et al., 1999; Puigserver et al., 2003). Therefore, we examined whether either FKHR (Foxo-1) or PGC-1 was functionally altered by L803-mts. Liver expression levels of FKHR were comparable in control and L803-mts-treated samples, as determined by simple Western blot analysis (Fig. 3C). In addition, phosphorylation of Foxo-1 at Ser²⁵⁶, which serves as indicator for inactivation of the protein (Biggs et al., 1999),

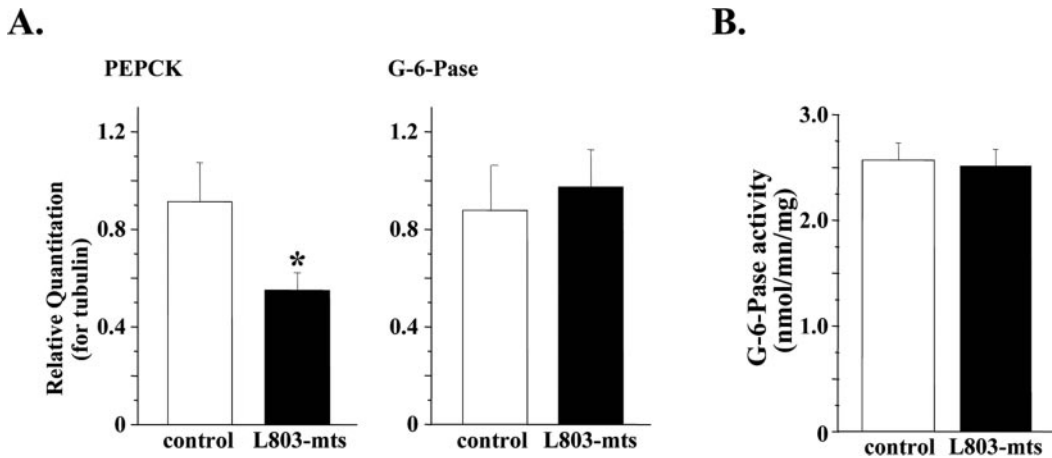


Fig. 2. Effect of L803-mts on PEPCK and G-6-Pase in the liver. A, mRNA expression levels of hepatic PEPCK and G-6-Pase were determined by quantitative real-time PCR as described under *Materials and Methods* and expressed as a relative amount of mRNA of tubulin $\beta 5$. Results are mean \pm S.E.M. of eight animals, *, $p < 0.05$. B, G-6-Pase activity was determined in liver extracts of control and L803-mts-treated animals as described under *Materials and Methods*. Results are the mean \pm S.E.M. of eight animals and presented as millimolar phosphate release per minute per milligram of protein tissues.

did not change by L803-mts (Fig. 3C). Subsequently, mRNA expression levels of PGC-1, determined by reverse transcription-PCR, were unchanged in L803-mts-treated samples (Fig. 3D).

IRS-2 Is Up-Regulated in Liver of L803-mts-Treated Animals. The next step was to observe the effect of L803-mts on insulin signal transduction. Livers were removed at the end of the treatment, and proteins were analyzed by immu-

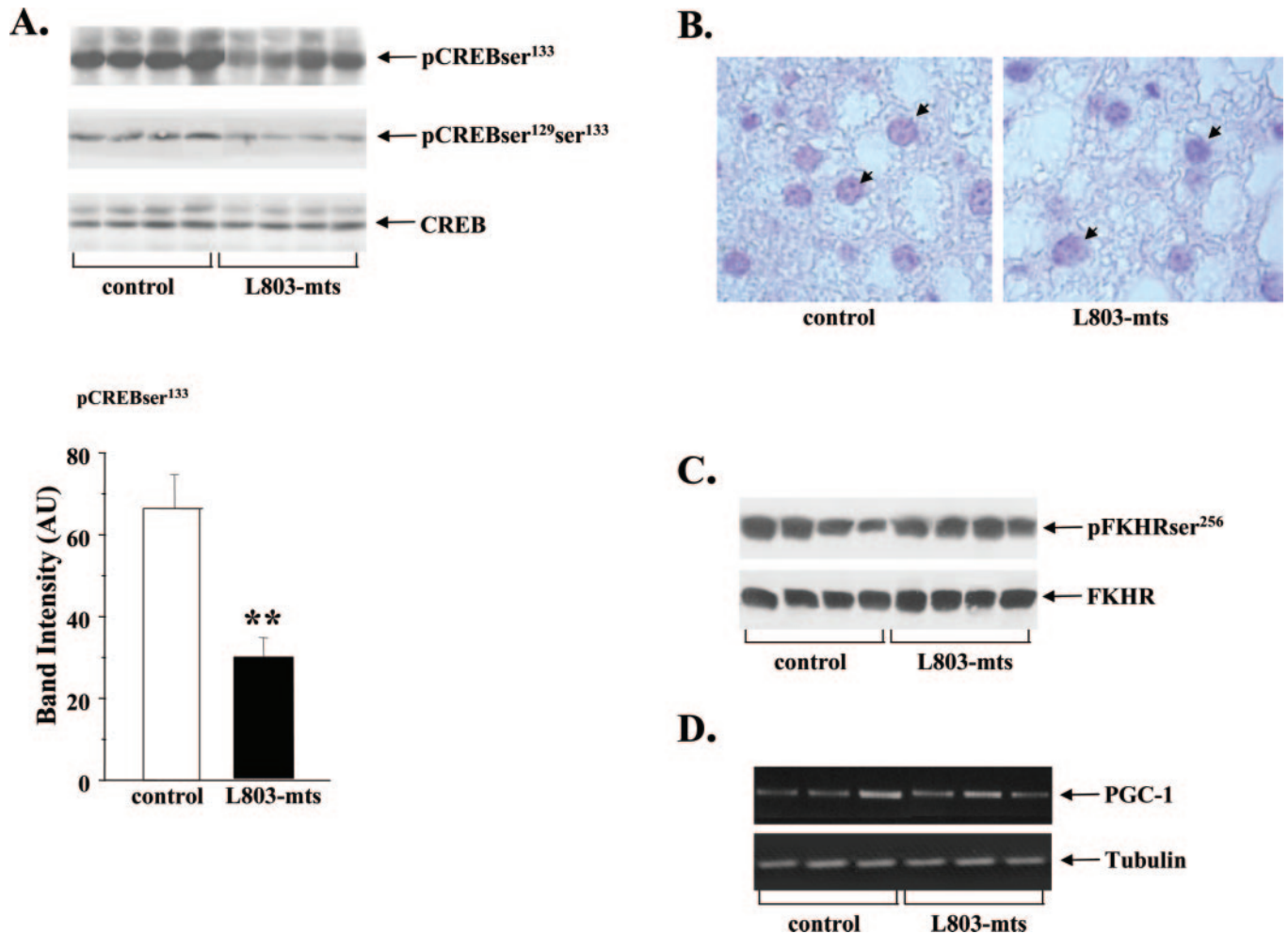


Fig. 3. CREB phosphorylation is suppressed by L803-mts. A, immunoblot analyses were performed with liver extracts prepared from control L803-mts-treated animals using anti-phospho-CREB¹³³, anti-phospho-Ser^{129,133}, or CREB antibody as indicated. Right panel shows the results of densitometry analysis of pCREB (Ser¹³³), which is a mean \pm S.E.M. of 19 to 22 liver samples. **, $p < 0.001$. B, immunohistochemistry against pCREB Ser¹³³ antibody of control and L803-mts-treated mice (shown original magnification, 40 \times). Note the loss of pCREB-positive in nuclei of the L803-mts-treated livers. C, immunoblot analyses were performed as A, with the exception that anti-FKHR antibody or anti-phospho-FKHR (Ser²⁵⁶) was used as indicated. Representative gel of four samples from 19 (control) or 22 (L803-mts treated) is shown. D, RNA samples were subjected to reverse transcription-PCR with PGC-1 or tubulin $\beta 5$ primers as described under *Materials and Methods*. Results present three samples of control or L803-mts-treated animals out of eight.

noblots analyses using specific antibodies (Fig. 4). Western blot analysis revealed no significant differences in the amount of IR, IRS-1, p85 α subunit of phosphatidylinositol 3-kinase, PKB, and GSK-3 α or β . In contrast, the amount of IRS-2 significantly increased in L803-mts-treated samples versus controls (Fig. 4, A and B), which was also associated with a small increase threonine 308 phosphorylation of its downstream target PKB (Fig. 4B). It is interesting to note that IRS-2 is largely reduced in the ob/ob liver (Shimomura et al., 2000). Treatment with L803-mts thus partially reversed this detrimental effect.

L803-mts Up-Regulates GLUT4 in Muscle. Given that muscle glucose transport and glycogen synthesis are severely impaired in ob/ob mice (Kato et al., 2004), we examined whether L803-mts affected these parameters in skeletal muscle. Western blot analysis of gastrocnemius muscle extracts revealed a marked increase in GLUT4 but not GLUT1 expression levels in L803-mts-treated samples (~2.5-fold) (Fig. 5A). Immunohistochemistry analysis further showed elevation of GLUT4 in the L803-mts-treated muscle (Fig. 5B). Because the staining procedure was performed in the absence of detergent, we assume that detected GLUT4 was mainly localized at the cell surface. We expected to see a correlation between increased GLUT4 and glycogen synthesis. Determination of glycogen levels revealed a 20% increase in glycogen from muscle of L803-mts-treated animals (0.50 ± 0.04 and 0.60 ± 0.03 mg/g tissue control versus L803-mts-treated, respectively, $n = 6$, *, $p < 0.05$). Immunoblot analyses were performed in muscle tissue extracts to determine the expression levels of insulin signaling components. As shown in Fig. 5C, there were no significant differences in IR, IRS-1, IRS-2, p85 α subunit of phosphatidylinositol 3-kinase, PKB, or GSK-3 isoforms.

Discussion

The discovery of GSK-3 as an important negative regulator of insulin signaling (Eldar-Finkelman and Krebs, 1997) had led to the idea that inactivation of GSK-3 may have therapeutic benefits in treatment of insulin resistance and type 2 diabetes. Previous work described the acute effect of selective GSK-3 inhibitors in both cells and animal models (Cline et al., 2002; Nikoulina et al., 2002; Henriksen et al., 2003; Plotkin et al., 2003). In the present work, we examined the long-term impact of in vivo inhibition of GSK-3 and investigated the molecular mechanisms of action of this treatment. Daily treatment of ob/ob mice with a selective GSK-3 peptide inhibitor L803-mts for 3 weeks reduced blood glucose levels, improved glucose tolerance, and prevented age-dependent increase of hyperglycemia. On the other hand, this treatment did not change body weight or food consumption. In addition, it was not associated with adverse effects, as judged by histopathology and blood chemistry analyses.

The improvement in glucose homeostasis prompted us to further investigate molecular events responsible for these effects in liver and muscle. We focused mainly on pathways controlling hepatic glucose production and muscle glucose transport. Our studies indicated that L803-mts attenuated gluconeogenesis and increased glycogen synthesis in the liver. The former was demonstrated by a 50% reduction in PEPCK gene expression in the liver of L803-mts-treated animals. This enzyme, which is a rate-determining step in gluconeogenesis, was shown to be sufficient to increase hepatic glucose production when overexpressed in animals (Valera et al., 1994; Rosella et al., 1995). It is also noteworthy that elevation in hepatic PEPCK is attributed mainly to accelerated gluconeogenesis and excessive HGP in ob/ob mice (Kreutner et al., 1975). We did not

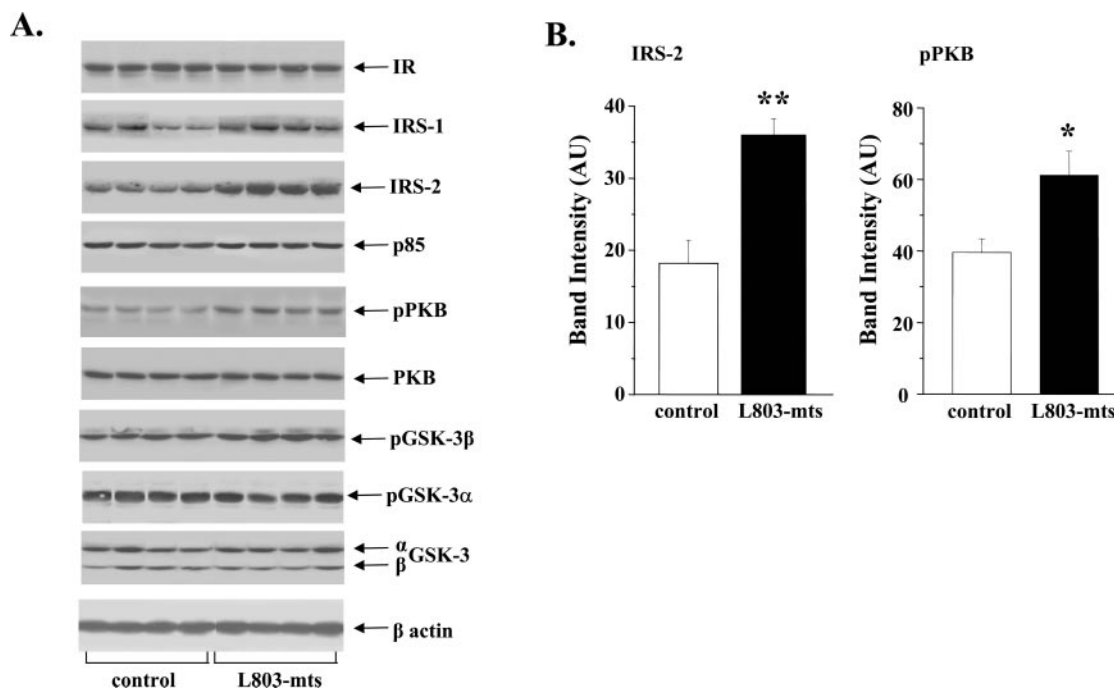


Fig. 4. Representative immunoblots and quantification of insulin-signaling proteins in L803-mts-treated livers. A, liver extracts of L803-mts-treated or control animals were subjected to immunoblot analyses with indicated antibodies. Tyrosine phosphorylation immunoblots of IRS-1 or IRS-2 (pIRS-1, pIRS-2) were performed after immunoprecipitation of the proteins with specific antibodies. Representative gels of 19 to 22 samples are shown. pPKB, phospho-PKB (Thr³⁰⁸); pGSK-3 α , phospho-GSK-3 (Ser²¹); pGSK-3 β , phospho-GSK-3 (Ser⁹). Densitometry analyses of IRS-2 protein or phosphorylated PKB (Thr³⁰⁸) are shown in B and are mean \pm S.E.M. of 19 (control) or 22 (L803-mts-treated) samples. **, $p < 0.001$; *, $p < 0.05$.

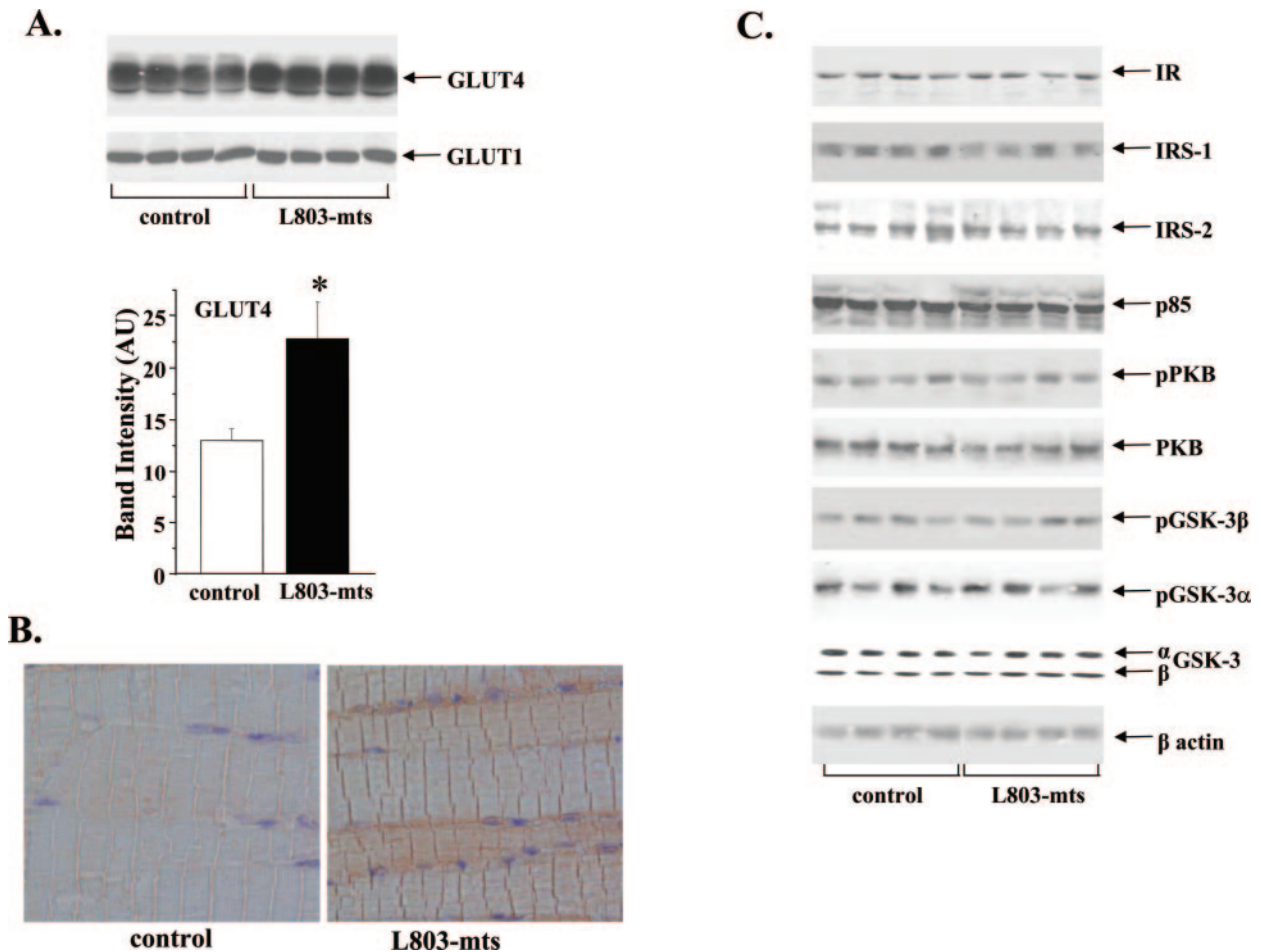


Fig. 5. Representative immunoblots and quantification of insulin-signaling proteins in L803-mts-treated muscle. A, tissue extracts of gastrocnemius muscle were subjected to immunoblotted analyses with specific antibody against GLUT4 or GLUT1. Representative gels of four samples from each group are shown. Lower panel shows densitometry analysis of GLUT4 bands representing a mean \pm S.E.M. of 19 (control) or 22 (L803-mts-treated) samples. *, $p < 0.05$. B, immunohistochemistry of GLUT4 in paraffin-embedded gastrocnemius muscle tissue sections using specific GLUT4 antibody indicates enhanced GLUT4 expression in L803-mts-treated animals (detected in brown). Sections were counterstained with hematoxylin to stain nuclei (purple) (initial magnification, 40 \times). C, tissue extracts of gastrocnemius muscle were subjected to immunoblotted analyses with indicated antibodies. pPKB, phospho-PKB (Thr³⁰⁸); pGSK-3 α , phospho-GSK-3 α (Ser²¹); pGSK-3 β , phospho-GSK-3 β (Ser⁹).

detect changes in liver G-6-Pase activity or its expression levels in the L803-mts-treated animals. This suggested that inhibition of GSK-3 selectively inhibited regulatory pathways upstream to PEPCK. This assumption may be correct, because different regulatory pathways and distinct DNA elements regulate PEPCK versus G-6-Pase (Hanson and Reshef, 1997; O'Brien et al., 2001). We cannot exclude the possibility, however, that inherent defects in the ob/ob liver can prevent suppression of G-6-Pase promoter activity.

The reduction in PEPCK gene expression suggested that one or more transcriptional activities are regulated by the treatment with L803-mts. The transcription factor CREB has been shown by numerous studies to stimulate PEPCK gene expression (Leahy et al., 1999; Herzig et al., 2001). In addition, its phosphorylation at Ser¹³³ has been shown to be essential for its transcriptional activity (Parker et al., 1996; Sassone-Corsi, 1998), and furthermore, phosphorylation at Ser¹³³ is increased in the ob/ob liver (Gum et al., 2003). Indeed, CREB-Ser¹³³ phosphorylation was reduced significantly in livers from L803-mts-treated animals, indicating that CREB activity was inhibited in these livers. Phosphorylation of CREB at Ser¹²⁹, which is also a direct target phosphorylated by GSK-3 (Fiol et al., 1994), decreased as well in livers from L803-mts-treated animals. The

latter provided evidence for in vivo inhibition of hepatic GSK-3 by L803-mts but also indicated for possible further inhibition of CREB (Fiol et al., 1994). Together, our studies show, for the first time, an in vivo role of GSK-3 in regulating hepatic CREB and explain the mechanisms by which inhibition of GSK-3 suppresses PEPCK. How inhibition of GSK-3 mediates phosphorylation of CREB at Ser¹³³ is not known at this time. It is possible that reduction in Ser¹²⁹ phosphorylation triggers dephosphorylation of Ser¹³³. Alternatively, inhibition of GSK-3 could inhibit CREB kinase(s) that phosphorylate Ser¹³³, such as PKA, PKC, p38, extracellular signal-regulated kinase, and calcium/calmodulin-dependent protein kinase (reviewed in Impey and Goodman, 2001). Current studies are trying to resolve this problem.

Hepatic insulin resistance is associated with the loss of IRS-2 (Withers et al., 1998). It is noteworthy that IRS-2 was up-regulated in L803-mts-treated animals, reversing the down-regulation effects observed in the ob/ob liver (Shimomura et al., 2000). The effect of L803-mts on IRS-2 was probably secondary and perhaps due to reduction in PEPCK. This last supposition is based on previous work showing that overexpression of hepatic PEPCK led to selective reduction in IRS-2, but not IRS-1 (Sun et al., 2002).

In muscle, our studies observed an increase in the protein levels of GLUT4. This was also associated with a 20% increase in glycogen levels indicating enhanced muscle glucose utilization in these animals. The precise mechanism responsible for up-regulation in GLUT4 is currently unknown at this point, but it should be noted that cross-talk between peripheral tissues may exist and thus improved hepatic metabolism could affect indirectly muscle glucose transport by yet unknown mechanisms.

Other studies also point toward the use of bioactive peptides as therapeutics approaches for target-selective pharmacotherapy. A great deal of progress has been made in recent years to produce cell-permeable peptides (Hawiger, 1999). Taking into consideration that L803-mts was selective toward GSK-3, stable in serum (Plotkin et al., 2003), and cell-permeable (Fig. 1A) (Plotkin et al., 2003) suggested its possible novel therapy for diabetes. It should be noted that GSK-3 is also involved in additional pathological disorders, such as bipolar disorder and Alzheimer's disease (Grimes and Jope, 2001; Gould et al., 2004), thus potential therapeutic use of L803-mts is yet to be explored, although it is most likely that the bioavailability of the peptide into the brain is limited.

In summary, we show that long-term inhibition of GSK-3 improved glucose homeostasis in ob/ob mice and provide evidence for a novel role of GSK-3 in regulating hepatic CREB activity and muscle GLUT4. Because gluconeogenesis and glucose transport are attractive targets for treatment of insulin resistance and type 2 diabetes, our studies suggest that inhibition of GSK-3 is instrumental in reducing hyperglycemia and reversing some of the diabetic phenotype.

Acknowledgments

We thank Batya Plotkin for performing glycogen synthase assays, Dr. Galina Wingarten for technical assistance in immunohistochemistry, Alina Cholostoy for helping in real-time PCR analyses, and Prof. Nava Bashan from Ben Gurion University for assisting in glycogen and G-6-Pase assays.

References

- Bailey CJ, Atkins TW, Conner MJ, Manley CG, and Matty AJ (1975) Diurnal variations of food consumption, plasma glucose and plasma insulin concentrations in lean and obese hyperglycaemic mice. *Horm Res* **6**:380–386.
- Biggs WH III, Meisenhelder J, Hunter T, Cavenee WK, and Arden KC (1999) Protein kinase B/Akt-mediated phosphorylation promotes nuclear exclusion of the winged helix transcription factor FKHR1. *Proc Natl Acad Sci USA* **96**:7421–7426.
- Cline GW, Johnson K, Regittng W, Perret P, Tozzo E, Xiano L, Damico C, and Shulman GI (2002) Effects of a novel glycogen synthase kinase-3 inhibitor on insulin-stimulated glucose metabolism in Zucker diabetic fatty (fa/fa) rats. *Diabetes* **10**:2903–2910.
- Coghlan MP, Culbert AA, Cross DA, Corcoran SL, Yates JW, Pearce NJ, Rausch OL, Murphy GJ, Carter PS, Roxbee Cox L, et al. (2000) Selective small molecule inhibitors of glycogen synthase kinase-3 modulate glycogen metabolism and gene transcription. *Chem Biol* **7**:793–803.
- Cohen P, Ntambi JM, and Friedman JM (2003) Stearoyl-CoA desaturase-1 and the metabolic syndrome. *Curr Drug Targets Immune Endocr Metabol Disord* **3**:271–280.
- Eldar-Finkelman H (2002) Glycogen synthase kinase-3: an emerging therapeutic target. *Trend Mol Med* **8**:126–132.
- Eldar-Finkelman H and Krebs EG (1997) Phosphorylation of insulin receptor substrate 1 by glycogen synthase kinase 3 impairs insulin action. *Proc Natl Acad Sci USA* **94**:9660–9664.
- Eldar-Finkelman H, Schreyer SA, Shinohara MM, LeBoeuf RC, and Krebs EG (1999) Increased glycogen synthase kinase-3 activity in diabetes- and obesity-prone C57BL/6J mice. *Diabetes* **48**:1662–1666.
- Fernandez-Novell JM, Arino J, and Guinovart JJ (1994) Effects of glucose on the activation and translocation of glycogen synthase in diabetic rat hepatocytes. *Eur J Biochem* **226**:665–671.
- Fiol CJ, Williams JS, Chou CH, Wang QM, Roach PJ, and Andrisani OM (1994) A secondary phosphorylation of CREB341 at Ser129 is required for the cAMP-mediated control of gene expression. A role for glycogen synthase kinase-3 in the control of gene expression. *J Biol Chem* **269**:32187–32193.
- Gould TD, Zarate CA, and Manji HK (2004) Glycogen synthase kinase-3: a target for novel bipolar disorder treatments. *J Clin Psychiatry* **65**:10–21.

- Grimes CA and Jope RS (2001) The multifaceted roles of glycogen synthase kinase 3beta in cellular signaling. *Prog Neurobiol* **65**:391–426.
- Gum RJ, Gaede LL, Heindel MA, Waring JF, Trevillyan JM, Zinker BA, Stark ME, Wilcox D, Jirousek MR, Rondinone CM, et al. (2003) Antisense protein tyrosine phosphatase 1B reverses activation of p38 mitogen-activated protein kinase in liver of ob/ob mice. *Mol Endocrinol* **17**:1131–1143.
- Hanson RW and Reshef L (1997) Regulation of phosphoenolpyruvate carboxykinase (GTP) gene expression. *Annu Rev Biochem* **66**:581–611.
- Hawiger J (1999) Noninvasive intracellular delivery of functional peptides and proteins. *Curr Opin Chem Biol* **3**:89–94.
- Henriksen EJ, Kinnick TR, Teachey MK, O'Keefe MP, Ring D, Johnson KW, and Harrison SD (2003) Modulation of muscle insulin resistance by selective inhibition of GSK-3 in Zucker diabetic fatty rats. *Am J Physiol* **284**:E892–E900.
- Herzig S, Long F, Jhala US, Hedrick S, Quinn R, Bauer A, Rudolph D, Schutz G, Yoon C, Puigserver P, et al. (2001) CREB regulates hepatic gluconeogenesis through the coactivator PGC-1. *Nature (Lond)* **413**:179–183.
- Impey S and Goodman RH (2001) CREB signaling—timing is everything. *Sci STKE* **82**:1–4.
- Kato M, Suwa A, and Shimokawa T (2004) Glucose catabolic gene mRNA levels in skeletal muscle exhibit non-coordinate expression in hyperglycemic mice. *Horm Metab Res* **36**:513–518.
- Kreutner W, Springer SC, and Sherwood JE (1975) Resistance of gluconeogenic and glycolytic pathways in obese-hyperglycemic mice. *Am J Physiol* **228**:663–671.
- Leahy P, Crawford DR, Grossman G, Gronostajski RM, and Hanson RW (1999) CREB-binding protein coordinates the function of multiple transcription factors including nuclear factor 1 to regulate phosphoenolpyruvate carboxykinase (GTP) gene transcription. *J Biol Chem* **274**:8813–8822.
- Liberman Z and Eldar-Finkelman H (2005) Serine 332 phosphorylation of insulin receptor substrate-1 by glycogen synthase kinase-3 attenuates insulin signaling. *J Biol Chem* **280**:4422–4428.
- Nakae J, Park BC, and Accili D (1999) Insulin stimulates phosphorylation of the forkhead transcription factor FKHR on serine 253 through a Wortmannin-sensitive pathway. *J Biol Chem* **274**:15982–15985.
- Nikoulina SE, Ciaraldi TP, Mudaliar S, Carter L, Johnson K, and Henry RR (2002) Inhibition of glycogen synthase kinase 3 improves insulin action and glucose metabolism in human skeletal muscle. *Diabetes* **51**:2190–2198.
- Nikoulina SE, Ciaraldi TP, Mudaliar S, Mohideen P, Carter L, and Henry RR (2000) Potential role of glycogen synthase kinase-3 in skeletal muscle insulin resistance of type 2 diabetes. *Diabetes* **49**:263–271.
- O'Brien RM, Streeper RS, Ayala JE, Stadelmaier BT, and Hornbuckle LA (2001) Insulin-regulated gene expression. *Biochem Soc Trans* **29**:552–558.
- Parker D, Ferreri K, Nakajima T, LaMorte VJ, Evans R, Koeber SC, Hoeger C, and Montminy MR (1996) Phosphorylation of CREB at Ser-133 induces complex formation with CREB-binding protein via a direct mechanism. *Mol Cell Biol* **16**:694–703.
- Plotkin B, Kaidanovich O, Talior I, and Eldar-Finkelman H (2003) Insulin mimetic action of synthetic phosphorylated peptide inhibitors of glycogen synthase kinase-3. *J Pharmacol Exp Ther* **305**:974–980.
- Puigserver P, Rhee J, Donovan J, Walkey CJ, Yoon JC, Oriente F, Kitamura Y, Altomonte J, Dong H, Accili D, et al. (2003) Insulin-regulated hepatic gluconeogenesis through FOXO1-PGC-1alpha interaction. *Nature (Lond)* **423**:550–555.
- Roach PJ (1991) Multisite and hierarchical protein phosphorylation. *J Biol Chem* **266**:14139–14142.
- Rosella G, Zajac JD, Baker L, Kaczmarczyk SJ, Andrikopoulos S, Adams TE, and Proietto J (1995) Impaired glucose tolerance and increased weight gain in transgenic rats overexpressing a non-insulin-responsive phosphoenolpyruvate carboxykinase gene. *Mol Endocrinol* **9**:1396–1404.
- Sassone-Corsi P (1998) Coupling gene expression to cAMP signalling: role of CREB and CREM. *Int J Biochem Cell Biol* **30**:27–38.
- Shimomura I, Matsuda M, Hammer RE, Bashmakov Y, Brown MS, and Goldstein JL (2000) Decreased IRS-2 and increased SREBP-1c lead to mixed insulin resistance and sensitivity in livers of lipodystrophic and ob/ob mice. *Mol Cell* **6**:77–86.
- Song XM, Fiedler M, Galuska D, Ryder JW, Fernstrom M, Chibalin AV, Wallberg-Henriksson H, and Zierath JR (2002) 5-Aminoimidazole-4-carboxamide ribonucleoside treatment improves glucose homeostasis in insulin-resistant diabetic (ob/ob) mice. *Diabetologia* **45**:56–65.
- Sun Y, Liu S, Ferguson S, Wang L, Klepcyk P, Yun JS, and Friedman JE (2002) Phosphoenolpyruvate carboxykinase overexpression selectively attenuates insulin signaling and hepatic insulin sensitivity in transgenic mice. *J Biol Chem* **277**:23301–23307.
- Thomas JA, Schlender KK, and Lerner J (1968) A rapid filter paper assay for UDP-glucose-glycogen glucosyltransferase, including an improved biosynthesis of UDP-14C-glucose. *Anal Biochem* **25**:486–499.
- Valera A, Pujol A, Pelegrin M, and Bosch F (1994) Transgenic mice overexpressing phosphoenolpyruvate carboxykinase develop non-insulin-dependent diabetes mellitus. *Proc Natl Acad Sci USA* **91**:9151–9154.
- Welsh GI and Proud CG (1993) Glycogen synthase kinase-3 is rapidly inactivated in response to insulin and phosphorylates eukaryotic initiation factor eIF-2B. *Biochem J* **294**:625–629.
- Withers DJ, Gutierrez JS, Towery H, Burks DJ, Ren JM, Previs S, Zhang Y, Bernal D, Pons S, Shulman GI, et al. (1998) Disruption of IRS-2 causes type 2 diabetes in mice. *Nature (Lond)* **391**:900–904.
- Woodgett JR and Cohen P (1984) Multisite phosphorylation of glycogen synthase. Molecular basis for the substrate specificity of glycogen synthase kinase-3 and casein kinase-II (glycogen synthase kinase-5). *Biochim Biophys Acta* **788**:339–347.

Address correspondence to: Dr. Hagit Eldar-Finkelman, Department of Human Genetics and Molecular Medicine, Sackler Institute of Molecular Medicine Sackler School of Medicine, Tel-Aviv University, Tel-Aviv 69978, Israel. E-mail: heldar@post.tau.ac.il