JPET Fast Forward. Published on March 6, 2003 as DOI:10.1124/jpet.102.047381

JPET/2002/47381

Insulin Mimetic Action of Synthetic Phosphorylated Peptide Inhibitors of Glycogen Synthase Kinase-3

Batya Plotkin, Oksana Kaidanovich, Ilana Talior and Hagit Eldar-Finkelman

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

JPET Fast Forward. Published on March 6, 2003 as DOI: 10.1124/jpet.102.047381
This article has not been copyedited and formatted. The final version may differ from this version.

Running Title- Novel GSK-3 inhibitors mimic insulin action

Corresponding author:

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

Tel: 972-3-6405307
Fax: 972-3-6405168
Email: heldar@post.tau.ac.il

Section: Molecular & Cellular

Pages: 25

Tables: 2

Figures: 6

References: 43

Abstract: 170 words

Introduction: 536 words

Discussion: 865 words

Abstract

Glycogen synthase kinase-3 (GSK-3) was shown to be a key factor in attenuation of the cellular action of insulin. We speculated that inhibition of GSK-3 might have a potential therapeutic value in treatment of insulin resistance and type 2 diabetes. Here we present a novel class of specific phosphorylated peptides inhibitors of GSK-3, that in sharp contrast to other protein kinase inhibitors, that are ATP analogs, are substrate competitive. We show that the GSK-3 peptide inhibitor activated glycogen synthase activity 2.5-fold in HEK 293 cells, and increased glucose uptake in primary mouse adipocytes in the absence or presence of insulin as compared to cells treated with two respective peptide controls. In addition, an intraperitoneal (i.p.) administration of GSK-3 peptide inhibitor to normal or insulin resistant obese C57Bl/6J mice, improved their performance on glucose tolerance tests compared to control treated animals.

We present here a novel rational strategy for developing specific GSK-3 inhibitors, and point toward GSK-3 as a promising therapeutic target in insulin resistance and type-2 diabetes.

Glycogen synthase kinase-3 (GSK-3) is a serine/threonine kinase that was originally identified by its ability to phosphorylate glycogen synthase, and later was found to be a key regulatory component of the insulin-signaling pathway (Cohen, 1986; Wang and Roach, 1993). We have recently demonstrated that GSK-3 is a key negative regulator of insulin action and have suggested that it is an important factor that contributes to insulin resistance and type-2 diabetes (Eldar-Finkelman and Krebs, 1997; Eldar-Finkelman et al., 1999). This concept was based on several lines of evidence. First, GSK-3 is constitutively active in unstimulated cells and is inhibited by insulin (Welsh and Proud, 1993; Cross et al., 1994), suggesting that the enzyme has a role in suppressing the insulin signal. Second, GSK-3 attenuates the cellular action of insulin via the phosphorylation of two important targets which are regulated by insulin- glycogen synthase and insulin receptor substrate-1 (IRS-1) (Cohen, 1986; Wang and Roach, 1993; Eldar-Finkelman and Krebs, 1997). Third, there is a marked decrease in glycogen synthase activity and in glycogen levels in muscle of type-2 diabetes patients (Shulman et al., 1990; Damsbo et al., 1991; Nikoulina et al., 1997). Finally, GSK-3 activity is elevated in fat and muscle tissues obtained from type 2 diabetic mice model and human patients respectively (Eldar-Finkelman et al., 1999; Nikoulina et al., 2000). Therefore, we thus proposed that specific GSK-3 inhibitors may therapeutically benefit in treating insulin resistance and type-2 diabetes.

Protein kinases are important targets for design and development of therapeutic drugs. Several approaches were used to develop protein kinase inhibitors such as high throughput screening of chemical libraries, structural biology based drug design and computer simulation (C. Gould and Wong, 2002; D. Williams and T. Mitchell, 2002; S. Sarno et al., 2002). Most of the protein kinase inhibitors developed so far are ATP competitive, and recently, pharmaceutical companies presented several ATP competitive inhibitors of GSK-3

and described their insulin like action in skeletal muscle cells and in Zucker Fa/Fa rats (Coghlan et al., 2000; Cline et al., 2002; Nikoulina et al., 2002). However, a major drawback of ATP- competitive inhibitors is their limited specificity, and therefore there is a concern that such inhibitors exert undesired side effects (Davies et al., 2000).

In contrast, substrate competitive inhibitors (which compete for the substrate binding site of the kinase) are more likely to be specific inhibitors. In the present study, we undertook to rationally develop substrate-competitive inhibitors for GSK-3, and to determine their insulin mimetic action *in vitro* and *in vivo*.

We hypothesized that small phosphorylated peptides derived from the unconventional recognition motif of GSK-3, namely $S^1XXXS^2(p)$ - where S^1 is the site phosphorylated by GSK-3, S^2 is the priming site, and X is any amino acid (Fiol et al., 1987; Zhang et al., 1993)) may serve as competitive inhibitors of GSK-3. We reasoned that since most protein kinases do not include a phosphorylated site as part of their recognition motif, such inhibitors would be very specific.

Here we show that synthetic phosphorylated peptides are substrate-specific competitive inhibitors of GSK-3 that mimic insulin action in *vitro* and *in vivo*. Thus, such phosphorylated peptides may serve as novel templates for the design of peptidomimetic drugs and small non-peptide molecules with important therapeutic implications.

Material and Methods

Material. Peptides were synthesized by Genemed Synthesis Inc (San Francisco, USA). Radioactive materials were purchased from Amersham Ltd. Cyclic dependent protein kinase, cdc2, casein kinase-2 (CK-2), CK-2 peptide , catalytic subunit of cAMP dependent protein kinase (PKA) and mitogen activated protein kinase (MAPK) were purchased from New England Bio-Labs (NJ, USA). All other reagents were from Sigma (Israel). Peptide inhibitors were dissolved in 50mM Hepes pH 7.5. Myristoylated peptides (mts) were dissolved in 0.1% DMSO buffer solution.

***In vitro* studies**

In vitro inhibition assays. Purified recombinant rabbit GSK-3β (Eldar-Finkelman et al., 1996) was incubated with peptide substrates PGS-1 (YRRAAVPPSPSLSRHSSPSQS(p)EDEEE) together with peptide inhibitor at indicated concentrations. The reaction mixture included Tris 50 mM pH 7.3, 10 mM MgAc, ^{32}P[γ-ATP] (100μM), 0.01% β-mercaptoethanol, and was incubated for 10 min at 30^{o}C. Reactions were spotted on phosphocellulose paper (p81), washed with 100 mM phosphoric acid, and counted for radioactivity as described (Eldar-Finkelman et al., 1996). The effect of L803 (200μM) on other protein kinases were tested. Cdc2 (1 unit) was incubated with a similar reaction mixture containing histone H1 substrate (5μg) and the reactions were boiled with SDS sample buffer, separated on gel electrophoresis and autoradiographed. MAPK, PKA and CK-2 activities were examined at similar conditions except that myelin basic protein (MBP, a gift from Zvi Naor), p9CREB (Table. 1), and CK-2 peptide were used as substrates respectively. Protein kinase C-δ was immunoprecipitated with a specific antibody (Santa Cruz, CA, USA) from fat tissue extracts and its activity was measured at similar conditions except that the lipid cofactor phosphatidylserine (40μM) was included together with histone H1 as a substrate. Protein

kinase B (PKB) was immunoprecipitated from extracts of serum-stimulated NIH/3T3 cells with a specific antibody (New-England Bio-labs, NJ, USA) and kinase assays were performed at similar conditions except that MBP was used as a substrate.

Graphics and statistical analyses were done by Origin 6.0 Professional software.

Glycogen synthase activity in HEK 293 cells To test the impact of peptide inhibitors in intact cells, we designed a membrane preamble L803 inhibitor **L803-mts** (N-Myristol-GKEAPPAPPQS(p)P) and two similarly modified respective controls LE803-mts ('N-Myristol-GKEAPPAPPQS**E**P) and LS803-mts (N-Myristol-GKEAPPAPPQ**S**P), in which phosphorylated serine was replaced with glutamic acid (that usually mimics a phosphorylated group) or a serine residue respectively. *In vitro* assays confirmed that the two peptides did not inhibit GSK-3. HEK 293 cells were grown in 10 cm plates with Dulbecco's modified Eagle medium (DMEM) supplemented with 10% foetal calf serum (FCS). On the day of the experiment, cells were incubated with low glucose medium supplemented with 0.5% FCS for 1 hr, followed by the addition of GSK-3 inhibitor L803-mts or its respective control LE803-mts and LS803-mts at various concentrations for additional 2.5 hr. A vehicle control of DMSO (0.1% DMSO) was also used. Cells were washed twice with ice cold GS buffer (50 mM Tris pH 7.8, 100 mM NaF, 10 mM EDTA + protease inhibitors: 20 μg/ml leupeptine, 10 μg/ml aprotinine, 10 mg/ml pepstatin A, 1 mM benzamidine), scraped with the same buffer, and frozen in liquid nitrogen as described (Eldar-Finkelman et al., 1996) Glycogen synthase activity was assayed according to the method of Thomas *et al* (Thomas et al., 1968) and based on the incorporation of uridine 5 -diphosphate [^{14}C] glucose (UDPG) into glycogen. Aliquots of cell lysates (15 μl) were incubated with 15 μl reaction mixture (66.6 mM Tris, pH 7.8, 32.5 mM KF, 0.8 μCi/μl [^{14}C]-UDPG (400μM), 13 mg/ml glycogen rabbit liver, Sigma)

for 20 min at 30^{o}C as described (Eldar-Finkelman et al., 1996). 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 0.1mM glucose-6-phosphate (G6P). Similar results were obtained when G6P was absent in the assays (data not shown).

Glucose uptake in isolated adipocytes Mice adipocytes were isolated from epididymal fat pad by digestion with 0.8 mg/ml collagenase (Worthington Biochemical, USA) as described previously (Lawrence et al., 1977). Digested fat pads were passed through nylon mesh and cells were washed 3 times with Krebs-bicarbonate buffer (pH 7.4) containing 1% bovine serum albumin (Fraction V, Boehringer Mannheim, Germany), 10 mM Hepes (pH 7.3), and 5 mM glucose and 200 nM adenosine. Cells were incubated with L803-mts or LE803-mts at indicated concentrations for 1 hr, followed by addition of 2-deoxy [^{3}H] glucose (0.5 μci/vial) for 10 min. The assay was terminated by centrifugation of cells through dinonylphthalate (ICN, USA), and ^{3}H was quantitated by liquid scintillation analyzer (Packard). Nonspecific uptake of 2-deoxy-[^{3}H] glucose was determined by the addition of cytochalasin B (50 μM) 30 min prior to the addition of radioactive material. In another set of experiments, adipocytes were treated with various concentrations of L803-mts 1 hr before addition of sub-optimal concentration of insulin (5 nM). Glucose uptake was determined as described above.

***In vivo* studies**

High fat diet-induced diabetes in animals- 4 weeks old C57Bl/6J mice received high fat diet containing 35% lard (Bioserve, Frenchtown, NJ) with 55% of calories from fat as previously described (Surwit et al., 1988). Animals were housed in individual cages with

free access to water in a temperature-controlled facility with 12h light/dark cycle. Animals developed obesity hyperglycemia and hyperinsulinemia after 16 weeks of diet feeding (manuscript submitted). Studies were done according to institutional animal care and use committee, and to Tel Aviv University animal care guidance.

Glucose tolerance tests and peptide stability test. Glucose tolerance tests were performed in overnight fasted C57Bl/6J mice (12 hr). L803-mts and LE803-mts were administrated intraperitoneally (i.p.) to mice (400nmol peptide) for 1 hr, glucose (1gr/kg) was injected then i.p., and blood samples were collected from tail vein at various time points. Blood glucose levels were immediately measured by Sugar Accutrend Sensor (Roche, Germany)). Similar experiments were performed in diabetic C57Bl/6J mice that were fed high fat dies for 16 weeks, except that mice fasted for 6 hr, and L803-mts was injected 90 min prior to glucose injection. The stability of L803-mts in serum was tested. L803-mts (1mM) was incubated with mouse serum (100µl or 200µl) for 2.5 hr in 37^{o}C. Primary adipocytes were incubated with the 'serum treated' L803-mts, L803-mts (10µM in 10µl each), or serum alone (10 µl), and glucose uptake was measured as described in previous section. Results indicated that the 'serum treated' L803-mts exhibited 88% activity of the maximal activity achieved with the 'non-treated' L803-mts.

JPET Fast Forward. Published on March 6, 2003 as DOI: 10.1124/jpet.102.047381
This article has not been copyedited and formatted. The final version may differ from this version.

Results

In vitro studies

The *in vitro* analyses were performed with purified GSK-3β. Table 1 lists the peptides used in the present studies. Replacement of S^1 with alanine in two known peptide sequences derived from GSK-3 substrates — CREB (cAMP responsive element binding protein) and HSF-1(heat shock factor-1) — converted the peptides into inhibitors (Table. 1, peptides # 5, 8). Replacement of the glutamic acid located upstream to S^1 in pAHSF peptide improved the potency of inhibition (L803, Table. 1, peptide#9).

Fig. 1A,B presents the inhibition curves of the 3 peptide inhibitors termed **Hz13**, **pAHS**F and **L803**, and indicates their IC_{50} values (range: 150 - 330 μM). The kinetic nature of the peptide inhibitors was studied by measuring the initial velocity as a function of the substrate concentration at several inhibitor concentrations. A Lineweaver -Burk plot of the GSK-3 inhibition by L803 indicated that L803 is a substrate-competitive inhibitor (Fig. 1C). Similar results were obtained for the other two peptides, Hz13 and pAHSF (data not shown).

As indicated in Table 1, glutamic acid, which often mimics a phosphorylated group, could not replace the phosphorylated serine in L803 peptide inhibitor (Table 1, peptides# 10 and 11), thus indicating that a phosphorylated serine is an absolute requirement for the peptide inhibitor. This conclusion is further demonstrated with the peptides derived from p9CREB substrate which lack the phosphorylated serine (peptides #3, and 4). Reducing the length of peptide inhibitor to the minimum sequence of SXXXS(p), also eliminated the inhibitory capacity of the peptide (Table 1 peptides #6, 12-14), which suggest that additional residues flanking this motif (apparently at least one at each end) must be included in the peptide inhibitor. As shown in Fig. 1, the inhibition was improved in

JPET Fast Forward. Published on March 6, 2003 as DOI: 10.1124/jpet.102.047381
This article has not been copyedited and formatted. The final version may differ from this version.

L803 peptide due to replacement of glutamic acid positioned upstream to S^1 with alanine (see peptide L803 versus pAHSF). Apparently, a glutamic residue is found in a similar position in some, (but not all), GSK-3 substrates, including eIF2B, CREB c- Myc, and D-Jun (Woodgett, 2001). This may point to a critical role for this residue in enzyme/substrate interaction and/ or dissociation. Further studies are needed to evaluate this point.

We expected the peptide inhibitors to be specific GSK-3 inhibitors because the determinant SXXXS(p) is not a part of the recognition motif of most protein kinases. We tested this assumption by examining the ability of several protein kinases to phosphorylate their substrates in the presence of the peptide inhibitors. Table 2 summarizes these results indicating the inability of L803 (200μM) to significantly inhibit a selection of protein kinases. Notably, the most closely related protein kinase to GSK-3, cycling dependent protein kinase (cdc2), was not inhibited by L803, further supporting the specificity of our inhibitor.

To test the biological effects of the peptide inhibitors in intact cells and in animals, we employed the myristoylated peptide L803-mts and two similarly modified respective controls LE803-mts and LS803-mts (see Material and Methods). *In vitro* kinetic analysis indicated that L803-mts inhibited purified GSK-3β (IC_{50} = 40μM, Fig. 2A), and behaved as a competitive inhibitor (Fig. 2B). Interestingly, L803-mts was a better inhibitor then L803, and this probably stems from the addition of the hydrophobic tail, which presumably improves the interaction of the peptide with the enzyme.

We first studied the effect of L803-mts on a known physiological target of GSK-3, glycogen synthase, which is inhibited upon phosphorylation by GSK-3 (Wang and

Roach, 1993). Studies of HEK 293 cells treated with either L803-mts, LE803-mts, or LS803-mts for 2.5 hr (Fig. 3A) indicated that L803-mts increased glycogen synthase activity by 2.5-fold as compared to cells treated with either LE803-mts or LS803-mts. *in vitro* kinetic analysis confirmed that LE803-mts or LS803-mts do not inhibit purified GSK-3 (Fig.3B). Altogether, L803-mts (10-40μM) inhibits endogenous GSK-3, indicating the L803-mts is a potent GSK-3 inhibitor in intact cells.

We next examined the impact of the GSK-3 inhibitors on glucose uptake in isolated adipocytes. Mouse adipocytes were incubated with either L803-mts or LE803-mts for 1 hr before measuring the uptake of [^{3}H]-2-deoxyglucose. As shown in Fig. 4A, L803-mts increased the incorporation of 2-deoxy- glucose by approximately 2.5-fold compared with cells treated with LE803-mts or LS803-mts. This value is comparable to that attained by maximum stimulation by insulin (10 nM), which is 3-fold in these mouse adipocytes (data not show). To determine whether our peptide GSK-3 inhibitor can work in concert with insulin, adipocytes were first treated with varied concentrations of L803-mts (1-10μM) followed by the addition of a sub-optimal concentration of insulin (5 nM). Activation of glucose uptake in the inhibitor-treated cells was further increased in insulin-treated cells (Fig. 4B). Thus, GSK-3 inhibitor L803-mts has an additive effect on insulin-induced glucose uptake.

To investigate the function of L803-mts *in vivo*, we measured glucose tolerance after i.p. administration of L803-mts in C57Bl/6J mice that had fasted overnight. Glucose was injected i.p into animals 1 hr following the administration of L803-mts or LE803-mts, and the blood glucose levels were monitored (Fig. 5). Glucose tolerance was better in fasted mice that were pretreated with the GSK-3 inhibitor L803-mts (Fig. 5) than in those treated with the control peptide. The former displayed a 20% reduction in the blood

glucose peak as well as a reduction in subsequent glucose levels 1 and 2 hr after glucose administration (Fig. 5). We next examined whether L803-mts improves glucose tolerance in diabetic mice. We used C57Bl/6J mice that develop obesity and insulin resistance upon high fat diet feeding (Surwit et al., 1988). Fig. 6 shows that when HF mice were pretreated with L803-mts, their performance on glucose tolerance testes significantly improved (34% reduction in the blood glucose peak) and blood glucose clearance was much faster to return to basal levels as compared to the animals treated with the control peptide LE803-mts.

JPET Fast Forward. Published on March 6, 2003 as DOI: 10.1124/jpet.102.047381
This article has not been copyedited and formatted. The final version may differ from this version.

Discussion

In this study, we present a new strategy for developing novel GSK-3 inhibitors. We show that phosphorylated peptides patterned after the unique recognition motif of GSK-3, namely SXXXS(p), are specific and competitive inhibitors. Thus, in contrast to other protein kinase inhibitors (including GSK-3 inhibitors reported by pharmaceutical companies) that are ATP competitive, our inhibitors are substrate competitive and most likely more specific towards GSK-3. Indeed, the GSK-3 peptide inhibitor L803-mts did not inhibit a selection of protein kinases including PKC, PKB, and cdc-2 protein kinase that is most closely related to GSK-3.

The initial observations that GSK-3 is inhibited by insulin (Welsh and Proud, 1993; Cross et al., 1994), and that lithium, a selective inhibitor of GSK-3 mimics insulin action (Cheng et al., 1983) suggested a possible link between GSK-3 and diabetes. Focusing on the potential role of the GSK-3 inhibitors in mimicking insulin action, we were able to show that GSK-3 peptide inhibitor L803-mts enhanced glycogen synthase activity in HEK 293 cells and promoted glucose uptake in primary mouse adipocytes. We also demonstrated a dose-dependent effect of the peptide inhibitors in cells treated with a sub-optimal concentration of insulin, indicating a potential additive effect of the GSK-3 inhibitor with insulin.

The GSK-3 inhibitors presented here may be used as novel tool for exploring biological consequences specifically effected by GSK-3. In this regard, our studies suggest a new role for GSK-3 in regulating glucose uptake. The precise molecular mechanism by which GSK-3 inhibition promotes glucose uptake is not fully understood at this point. It could be that the phosphorylation of IRS-1 by GSK-3 interferes with its ability to recruit and activate PI3 kinase, apparently a key factor in activation of the GLUT4 glucose transporter translocation to the cell surface (Katagiri et al., 1996; Ricort et al., 1996) and

in the enhancement of insulin sensitivity (Mauvais-Jarvis et al., 2002; Ueki et al., 2002). A different mechanism may be used by GSK-3 to promote glucose uptake via its ability to phosphorylate and inactivate the kinesin protein (Morfini et al., 2002), which was recently found to be involved in the regulation of membrane trafficking of GLUT4 vesicles to the plasma membrane (Emoto et al., 2001). Whatever the mechanism, the intriguing link between GSK-3 inhibition and enhancement of glucose uptake points GSK-3 as a promising therapeutic target for insulin resistance and type-2 diabetes, and current studies explore the possible pathways that link GSK-3 with glucose uptake.

Development of bioactive peptides as therapeutics offers exciting approaches for target-selective pharmacotherapy. Notably, nature itself has selected peptides as the finest and most widespread anti-microbial agents of numerous invertebrates as well as certain vertebrates (Zasloff, 2002). The challenging task in peptide therapy is the delivery route, namely, introduction of peptides across cell membranes and the optimization of peptide delivery and bioavailability. A great deal of progress has been made in recent years, to produce cell –permeable peptides (Hawiger, 1999; Tung and Weissleder, 2002; Lindsay, 2002) as well as the use of alternative delivery routes such as bucul and nasal routes (Heinemann et al., 2001; Senel et al., 2001). Still, the *in vivo* stability of peptides and their bioavailability are important issues in this field. Recent studies, however, indicated that these problemss might be improved. It has been demonstrated that association of peptides with albumin, the most abundant protein in plasma, significantly prolonged their half-life in the blood stream, and dramatically improved their *in vivo* phramacokinetics properties (Markussen et al., 1996; Dennis et al., 2002). It was further shown that formation of peptide/albumin complex also limited renal clearance of peptides, because kidney generally filters out molecules below 60kDa (Dennis et al., 2002). Importantly,

conjugation of peptides with fatty acids was shown to facilitate the peptide binding with albumin, and hence, improved their stability *in vivo* (Markussen et al., 1996), and limited their filtration by the kidney (Wang et al., 2002). The fatty acid-conjugated peptides were also shown to be more stable in liver as compared with their respective non-lipidated peptides (Wang et al., 2002). It is therefore suggested that L803-mts (which is conjugated to fatty acid), pocess similar properties to that described for the lipidated peptides (Markussen et al., 1996; Covic et al., 2002; Wang et al., 2002), namely, it is stable in serum, and is not rapidly destructed by kidney or liver. Our studies partially supported this notion by showing that L803-mts was not degraded in serum (see material and methods). The fine equilibrium between the peptide-albumin-bound and the 'free' peptide is another important factor, which determines the peptide availability (Rowald, 1988). It is suggested from our *in vivo* experiments, that sufficient concentrations of 'free' L803-mts were available to tissues. The specific localization of L803-mts in the animal tissues was not determined, however, it may be concluded from the GTT experiments (Fig. 5&6), that efficient concentrations of L803-mts were accumulated in target tissues such as muscle and fat to promote their glucose uptake. Previous studies demonstrated that peptides can be delivered into tissues *in vivo*: a 15- oligomer peptide attached to protein transduction domain (PTD) of the human immunodeficiency virus TAT protein, was detected in blood cells, skeletal muscle and brain tissue after i.p. injection into mice (Schwarze et al., 1999), another study indicated that fluorescent-labeled palmitoylated peptides were acquired in mouse platelets after their injection into the animals (Covic et al., 2002). Thus, lipidated peptides have the ability to penetrate into the body cells (Covic et al., 2002), nevertheless, the precise tissues' distribution of L803-mts remains to be elucidated.

In summary, GSK-3 peptide inhibitors presented here may be conceived as useful compounds, alternatively they may serve as novel templates for the design of peptidomimetic drugs and small non-peptide molecules targeting GSK-3.

Acknowledgment

We would like to thank Professor Shmuel Ben-Sasson from The Hebrew University of Jerusalem for his help in designing the mts-peptides and the *in vivo* experiments, and to Dr. David Gurwitz from the National Laboratory for the Genetics of Israeli Populations in Tel Aviv University for critical review and discussions of this manuscript.

References

Gould C and Wong CF (2002) designing specific protein kinase inhibitors: insights from computer simulations and comparative sequence/structure analysis. *Pharamacology & Theraputics* **93**:1690178.

Tung C-H and Weissleder R (2002) Arginine containing peptides as delivery vectors. *Adv. Drug Deliv. Rev*. **in press**.

Cheng K, Creacy S and Larner J (1983) 'Insulin-like' effects of lithium ion on isolated rat adipocytes. II. Specific activation of glycogen synthase. *Mol. .Cell. Biochem.* **56**:183-189.

Cline GW, Johnson K, Regittnig 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*:2903-2910.

Coghlan MP, Culbert AA, Cross DA, Corcoran SL, Yates JW, Pearce NJ, Rausch OL, Murphy GJ, Carter PS, Roxbee Cox L, Mills D, Brown MJ, Haigh D, Ward RW,

Smith DG, Murray KJ, Reith AD and Holder JC (2000) Selective small molecule inhibitors of glycogen synthase kinase-3 modulate glycogen metabolism and gene transcription. *Chemistry & Biology* **7**:793-803.

Cohen P (1986) *Muscle glycogen synthase, The enzymes, edited by Boyer. P, and Krebs, E. G.* Academic Press, Orlando, FL.

Covic L, Misra M, Badar J, Singh C and Kuliopulos A (2002) Pepducin-based intervention of thrombin-receptor signaling and systemic platelet activation. *Nature Med.* **10**:1161-1165.

Cross DA, Alessi DR, Vandenheede JR, McDowell HE, Hundal HS and Cohen P (1994) The inhibition of glycogen synthase kinase-3 by insulin or insulin-like growth factor-1 in the rat skeletal muscle cell line L6 is blocked by wortmannin, but not by rapamycin: evidence that wortmannin blocks activation of the mitogen-activated protein kinase pathway in L6 cells between Ras and Raf. *Biochem. J.* **303**:21-26.

Williams D and T. Mitchell (2002) Latest development in crystallography and structure-based design pf protein kinase inhibitors as drug candidates. *Curr. Opin. Pharmacol.* **2**:567.

Damsbo P, Vaag A, Hother-Nielsen O and Beck-Nielsen H (1991) Reduced glycogen synthase activity in skeletal muscle from obese patients with and without type 2 (non-insulin-dependent) diabetes mellitus. *Diabetologia* **34**:239-245.

Davies SP, Reddy H, Caivano M and Cohen P (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochemical Journal* **351**:95-105.

Dennis MS, Zhang M, Meng YG, Kadkhodayan M, Kirchhofer D, Combs D and Damico LA (2002) Albumin binding as a general strategy for improving the pharmacokinetics of proteins. *J. Biol. Chem.* **277**:35035-35043.

Eldar-Finkelman H, Agrast GM, Foord O, Fischer EH and Krebs EG (1996) Expression and Characterization of glycogen synthase kinase-3 mutants and their effect on glycogen synthase activity. *Proc. Natl. Acad. Sci. USA* **93**:10228-10233.

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.* **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.

Emoto M, Langille SE and Czech MP (2001) A role for kinesin in insulin-stimulated GLUT4 glucose transporter translocation in 3T3-L1 adipocytes. *J. Biol. Chem.* **276**:10677-10682.

Fiol CJ, Mahrenholz AM, Wang Y, Roeske RW and Roach PJ (1987) Formation of protein kinase recognition sites by covalent modification of the substrate. Molecular mechanism for the synergistic action of casein kinase II and glycogen synthase kinase 3. *J. Biol. Chem.* **262**:14042-14048.

Hawiger J (1999) Noninvasive intracellular delivery of functional peptides and proteins. *Curr. Opin. Chem. Biol.* **3**:89-94.

Heinemann L, Pfutzner A and Heise T (2001) Alternative routes of administration as an approach to improve insulin therapy: update on dermal, oral,nasal and pulmonary delivery. *Curr. Pharm. Des.* **14**:1327-1351.

Katagiri H, Asano T, Ishihara H, Inukai K, Shibasaki Y, Kikuchi M, Yazaki Y and Oka Y (1996) Overexpression of catalytic subunit p110alpha of phosphatidylinositol 3-kinase increases glucose transport activity with translocation of glucose transporters in 3T3-L1 adipocytes. *J Biol Chem* **271**:16987-16990.

Lawrence JC, Guinovart JJ and Larner J (1977) Activation of Rat Adipocyte Glycogen Synthase by Insulin. *J. Biol. Chem.* **252**:444-450.

Lindsay MA (2002) Peptide-mediated cell delivery: application in protein target validation. *Curr. Opin. Pahrmacol.* **2**:587-594.

Markussen J, Havelund S, Kurtzhals P, Andersen AS, Halstrom J, Hasselager E, Larsen UD, Ribel U, Schaffer L, Vad K and Jonassen I (1996) Soluble, fatty acid acylated insulins bind to albumin and show protracted action in pigs. *Diabetologia* **39**:281-288.

Mauvais-Jarvis F, Ueki K, Fruman DA, Hirshman MF, Sakamoto K, Goodyear LJ, Iannacone M, Accili D, Cantley LC and Kahn CR (2002) Reduced expression of the murine p85alpha subunit of phosphoinositide 3-kinase improves insulin signaling and ameliorates diabetes. *J. Clin. Invest.* **109**:141-149.

Morfini G, Szebenyi G, Elluru R, Ratner N and Brady ST (2002) Glycogen synthase kinase 3 phosphorylates kinesin light chains and negatively regulates kinesin-based motility. *EMBO J.* **21**:281-293.

Nikoulina SE, Ciaraldi- TP, Abrams-Carte L, Mudaliar S, Park KS and Henry RR (1997) Regulation of glycogen synthase activity in cultured skeletal muscle cells from subjects with type II diabetes: role of chronic hyperinsulinemia and hyperglycemia. *Diabetes* **46**:1017-1024.

Nikoulina SE, Ciaraldi TP, Mudailar S, Mohideen P, Carter L and Henry RR (2000) Potential role of GSK-3 in skeletal muscle insulin resistance type 2 diabetes. *Diabetes* **49**:263-271.

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.

Ricort JM, Tanti JF, Van Obberghen E and Le Marchand-Brustel Y (1996) Different effects of insulin and platelet-derived growth factor on phosphatidylinositol 3-kinase at the subcellular level in 3T3-L1 adipocytes. A possible explanation for their specific effects on glucose transport. *Eur. J. Biochem.* **239**:17-22.

Rowald M, (1988) Clinical Pharmacokinetics: Concepts and Applications. **2nd Ed**:Lea&Febiger, Philadelphia, PA.

Sarno S, Moro, F. Meggio, G. Zagotto, D. Dal Ben, P. Ghisellini, R. Battistutta, G. Zanotti and L.A. Pinna (2002) Toward the rational design of protein kinase casein kinase-2 inhibitors. *Pharmacology & Therapeutics* **93**:159-168.

Schwarze SR, A. Ho, A. Vocero-Akbani and Dowdy SF (1999) In vivo protein transduction: delivery of a biologically active protein into the mouse. *Science* **285**:1569-1572.

Senel S, Kremer M, Nagy K and Squier C (2001) Delivery of Bioactive peptides and proteins across oral (buccal) mucosa. *Curr. Pharm. Biotechnol.* **2**:175-186.

Shulman GI, Rothman DL, Jue T, Stein P, DeFronzo RA and Shulman RG (1990) Quantitation of muscle glycogen synthesis in normal subjects and subjects with non-insulin-dependent diabetes by 13C nuclear magnetic resonance spectroscopy. *N. Engl. J. Med.* **22**:223-228.

Surwit RS, Kuhn CM, Cochrane C, McCubbin JA and Feinglos MN (1988) Diet-Induced Type II Diabetes in C57BL/6J Mice. *Diabetes* **37**:1163-1167.

Thomas JA, Schlender KK and Larner J (1968) A rapid filter paper assay for UDPglucose-glycogen glucosyltransferase, including an improved biosynthesis of UDP-14C-glucose. *Anal. Biochem.* **25**:486-499.

Ueki K, Yballe CM, Brachmann SM, Vicent D, Watt JM, Kahn CR and Cantley LC (2002) Increased insulin sensitivity in mice lacking p85beta subunit of phosphoinositide 3-kinase. *Proc. Natl. Acad. Sci. USA* **99**:419-424.

Wang J, Wu d and Shen W-C (2002) Structure-activity relationship of reversible lipidized peptides: studies of fatty acid-desmopression conjugates. *Pharmacuet. Res.* **19**:609-614.

Wang Y and Roach PJ (1993) Inactivation of rabbit muscle glycogen synthase by glycogen synthase kinase-3. Dominant role of the phosphorylation of Ser-640 (site-3a). *J. Biol. Chem.* **268**:23876-23880.

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.

Woodgett JR (2001) Judging a protein more than its name: GSK-3. *Sci. STKE* **100**:RE12.

Zasloff M (2002) Antimicrobial peptides of multicellular organisms. *Nature* **415**:389-395.

Zhang W, Depaoli-Roach AA and Roach PJ (1993) Mechanisms of multisite phosphorylation and inactivation of rabbit muscle glycogen synthase. *Arch. Biochem. Biophys.* **304**:219-225.

JPET Fast Forward. Published on March 6, 2003 as DOI: 10.1124/jpet.102.047381
This article has not been copyedited and formatted. The final version may differ from this version.

Figure Legends

Fig. 1 *In vitro* kinetic analysis of phosphorylated peptide inhibitors. The ability of GSK-3 to phosphorylate PGS-1 peptide substrate was measured in the presence of indicated concentrations of peptides HZ13 (**A**), pAHSF and L803 (**B**). Results represent the percentage of GSK-3 activity in control incubation in which peptide inhibitors were omitted. Results are mean of 3 independent experiments ±SEM, where each point was assayed in triplicate. IC50 Hz13=330 μM, pAHSK=300μM, and L803=150 μM. **C.** Lineweaver Burk plot of inhibition of GSK-3 by L803 at indicated concentrations. Results represent phosphate incorporation into PGS-1 peptide substrate (CPM). Results show one representative experiment out of 4**.** Each point is a mean of duplicated sample. Calcuated Ki=70 μM±10.

Fig. 2 *In vitro* kinetic analysis of L803-mts. **A.** The ability of GSK-3 to phosphorylate PGS-1 peptide substrate was measured in the presence of indicated concentrations of L803-mts. Results represent the percentage of GSK-3 activity in control incubation in which peptide inhibitors were omitted. Results are mean of 2 independent experiments ±SEM, where each point was assayed in triplicate. **B.** Lineweaver Burk plot of inhibition of GSK-3 by L803 at indicated concentrations. Results represent phosphate incorporation into PGS-1 peptide substrate (CPM). Results show one representative experiment out of 3. Each point is a mean of duplicated sample.

Fig. 3 L803-mts is a potent GSK-3 inhibitor in intact cells. HEK293 cells were treated with L803-mts and control peptide LE803-mts or LS803-mts for 2.5 hr at indicated concentrations. Lysate supernatants were assayed for glycogen synthase activity. The activity of glycogen synthase in cells treated with vehicle DMSO (0.1% DMSO) was normalized to 1 unit and the values for glycogen synthase activity observed in cells treated

with GSK-3 inhibitor (filled circles) and its respective controls (LE803mts- hollow circles, LS803-mts- circles with x) are presented as fold stimulation over the cells treated with vehicle DMSO. Data are mean of 3 independent experiments ±SEM where each point was assayed in duplicate. *, value significantly greater than cells treated with control peptides **B**. The inhibition of purified GSK-3β by L803-mts but not by control peptides LE803-mts or LS803-mts (50μM each) is shown. *In vitro* kinase assays were performed as described in legend to Fig. 1.

Fig. 4 GSK-3 peptide inhibitor increases glucose uptake in the absence or presence of insulin. **A**. Adipocytes were isolated from mouse epididymal fat tissue and incubated with L803-mts for 75 min. Glucose uptake into cells was assayed with [^{3}H] 2-deoxy glucose as described in Material and Methods. The relative [^{3}H] 2-deoxy glucose incorporation observed in adipocytes treated with LE803-mts was normalized to 1 unit and the values obtained for [^{3}H] 2-deoxy glucose in adipocytes treated with L803-mts are presented as fold activation over cells treated with the peptide control, and are the mean of 5 independent experiments ± SEM, where each point was assayed in triplicate. *,value significantly greater than control. **B.** Adipocytes were treated with or without L803-mts at indicated concentrations for 30 min and then insulin (5nM) was added for another hour. Glucose uptake into cells was assayed with [^{3}H] 2-deoxy glucose as described for **A**. Results are presented as fold activation of glucose uptake in cells treated with L803-mts over cells treated with insulin (normalized as 1 unit), and are an average of 4 experiments ± SEM, where each point was assayed in triplicate. *, value significantly greater than cells treated with insulin.

Fig. 5 . **GSK-3 inhibitor improves glucose tolerance.** Following overnight fasting, animals were injected i.p. with L803-mts or LE803-mts 1 hr prior to injection of glucose. Blood glucose (mg/dL) levels were measured at the indicated time points. Results are mean of 12

animals treated with L803-mts (filled circles) or 9 animals treated with control peptide LE803-mts (hollow circles) ±SEM. *, value significantly less than animals treated with control peptide.

Fig. 6 GSK-3 inhibitor improves glucose tolerance in diabetic mice. **A.** Following 6 hr fasting, HF mice were injected i.p. with L803-mts (filled circles) or LE803-mts (hollow circles) 90 min prior injection of glucose (1gr/kg) and blood glucose levels were measured at the indicated time points. Results present the mean of 10 animals ±SEM. **B**. Results present the percent of the blood glucose levels measured at the time of glucose injection (time=0). *, value significantly less than animals treated with control peptide.

JPET Fast Forward. Published on March 6, 2003 as DOI: 10.1124/jpet.102.047381
This article has not been copyedited and formatted. The final version may differ from this version.

Peptide	Lenght	Substrate	Inhibitor	Comments
1.KRREIL**S**[1]RRP**S**[2](p)YR	13	+	-	Derived from CREB
2. ILSRRPS(p)YR	9	+		**p9CREB**
3. ILSRRPEYR	9	-	-	
4. ILSRRPSYR	9	-	-	
5. KRREIL**A**RRPS(p)YR	13	-	+	**Hz13**
6. IL**A**RRPS(p)YR	10	-	*	
7. KEEPP**S**PPQS(p)P	11	+	-	Derived from Heat shock factor-1
8. KEEPP**A**PPQS(p)P	11	-	+	**pAHSF**
9. KE**A**PP**A**PPQS(p)P	11	-	+	**L803**
10. KEEPP**A**PPQSP	11	-	-	
11. KEEPP**A**PPQEP	11	-	-	
12. P**A**PPQS(p)P	7	-	*	
13. EPP**A**PRRE	8	-	-	
14. EPP**A**PR	6	-	-	

Table. 1 peptide sequences used in these studies. The property of peptide as substrate or inhibitor (or neither of them) is indicated. S^1 and S^2 are marked bold in peptide substrate #1. Replacement of S^1 in other peptides is marked bold. S(p) represents phosphorylated serine. * weak inhibition (IC50>800μM).

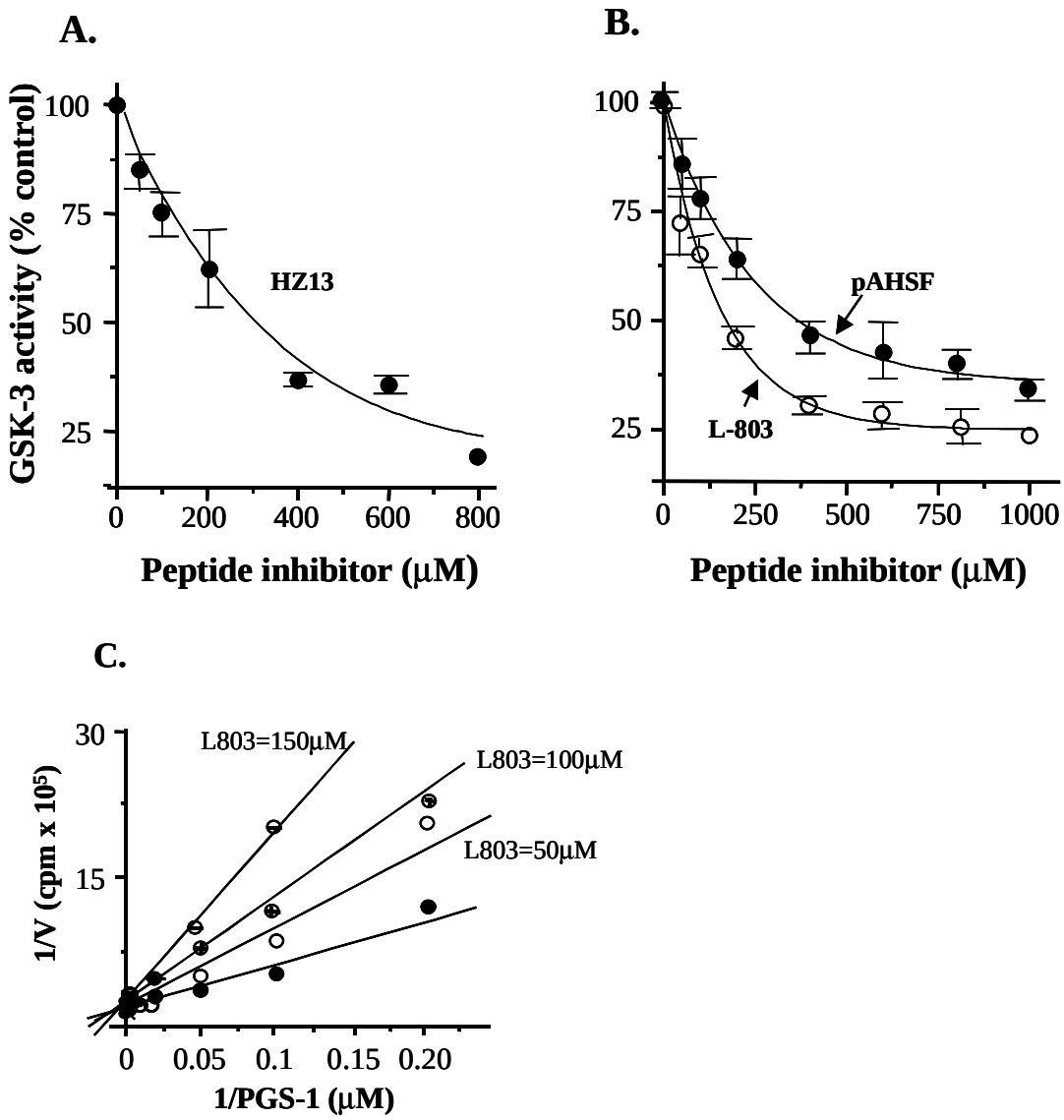
JPET Fast Forward. Published on March 6, 2003 as DOI: 10.1124/jpet.102.047381
This article has not been copyedited and formatted. The final version may differ from this version.

Protein kinase	% Maximal activity
MAPK	106
PKA	86
CK-2	117
Cdc2	90
PKC-δ	111
PKB	91

Table 2 L803 inhibitor does not inhibit other protein kinases. Protein kinase assays were performed in the presence or absence of 200 μM L803 as described in material and methods. Results present the percent of the activity measured in the absence of the inhibitor.

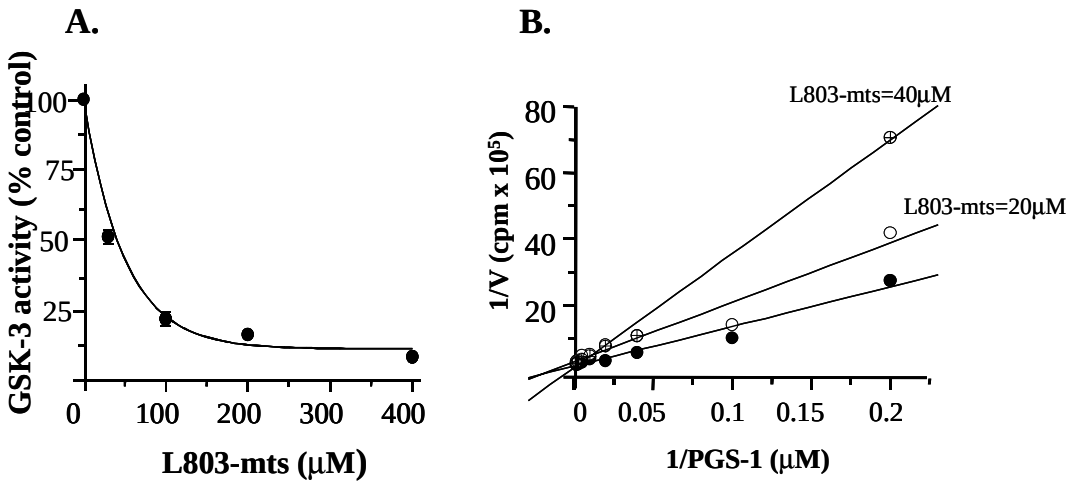
JPET Fast Forward. Published on March 6, 2003 as DOI: 10.1124/jpet.102.047381
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 1



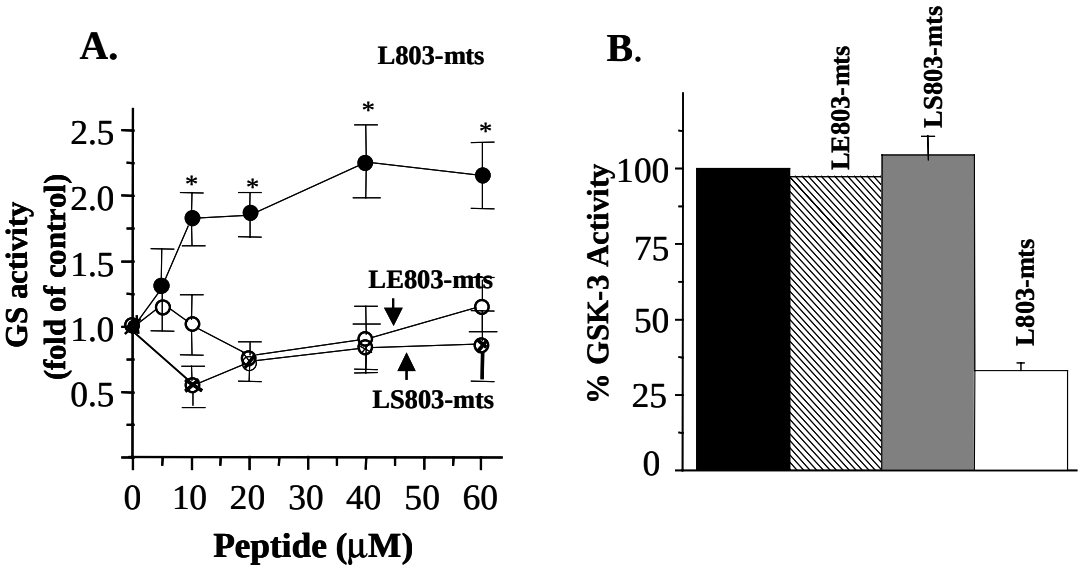
JPET Fast Forward. Published on March 6, 2003 as DOI: 10.1124/jpet.102.047381
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 2



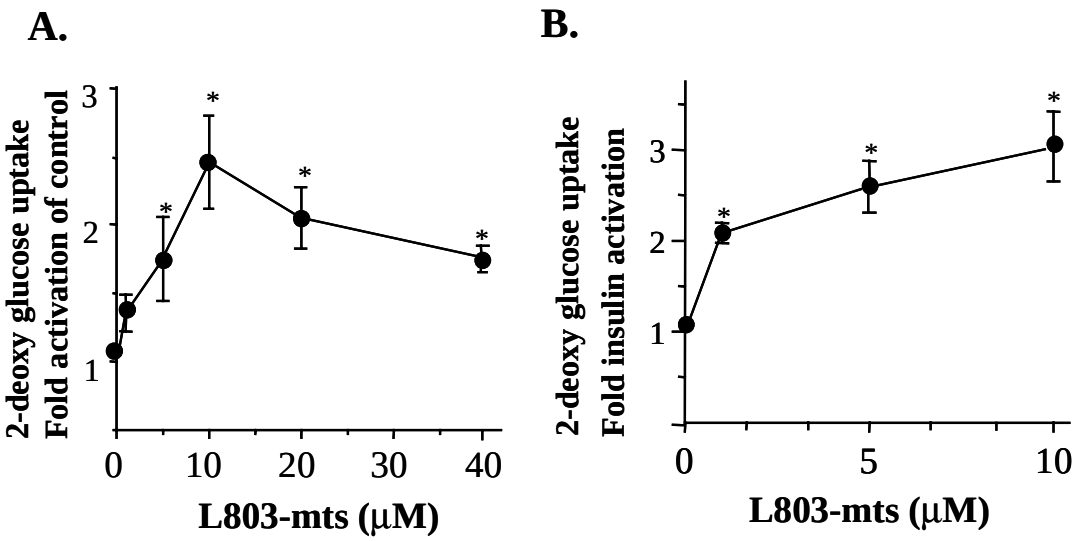
JPET Fast Forward. Published on March 6, 2003 as DOI: 10.1124/jpet.102.047381
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 3



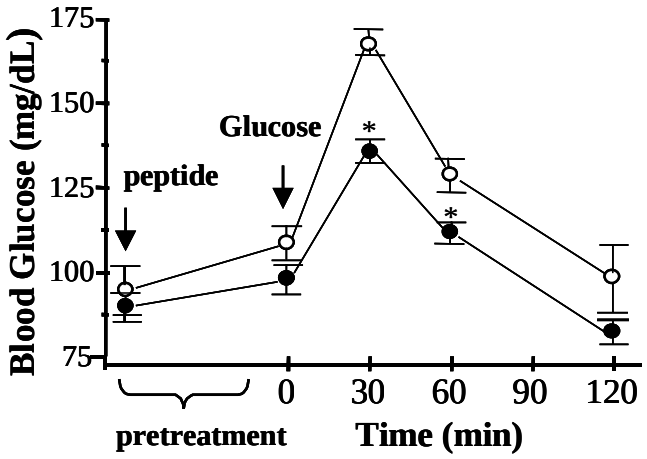
JPET Fast Forward. Published on March 6, 2003 as DOI: 10.1124/jpet.102.047381
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 4



JPET Fast Forward. Published on March 6, 2003 as DOI: 10.1124/jpet.102.047381
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 5



JPET Fast Forward. Published on March 6, 2003 as DOI: 10.1124/jpet.102.047381
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 6