

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

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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, which 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 human embryonic kidney 293 cells, and

increased glucose uptake in primary mouse adipocytes in the absence or presence of insulin compared with cells treated with two respective peptide controls. In addition, an 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 with 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 that are regulated by insulin-glycogen synthase and insulin receptor substrate-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 pa-

tients, 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 (Gould and Wong, 2002; Sarno et al., 2002; Williams and Mitchell, 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,

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ABBREVIATIONS: GSK-3, glycogen synthase kinase-3; CK-2, casein kinase-2; PKA, protein kinase A; mts, myristoylated peptides; PKB, protein kinase B; DMSO, dimethyl sulfoxide; HEK, human embryonic kidney; CREB, cAMP-responsive element binding protein; HSF-1, heat shock factor-1.

namely, S¹XXXS²(p), where S¹ is the site phosphorylated by GSK-3, S² 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 because 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 nonpeptide molecules with important therapeutic implications.

Materials and Methods

Material

Peptides were synthesized by Genemed Synthesis (San Francisco, CA). Radioactive materials were purchased from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK). 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 were purchased from New England Biolabs (Beverly, MA). All other reagents were from Sigma-Aldrich (St. Louis, MO). Peptide inhibitors were dissolved in 50 mM 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-3 β (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 50 mM Tris, pH 7.3, 10 mM MgAc, 100 μ M [γ -³²P]ATP, and 0.01% β -mercaptoethanol and was incubated for 10 min at 30°C. Reactions were spotted on phosphocellulose paper (p81), washed with 100 mM phosphoric acid, and counted for radioactivity as described previously (Eldar-Finkelman et al., 1996). The effect of L803 (200 μ M) on other protein kinases was tested. cdc2 (1 unit) was incubated with a similar reaction mixture containing histone H1 substrate (5 μ g), and the reactions were boiled with SDS sample buffer, separated on gel electrophoresis, and autoradiographed. Mitogen-activated protein kinase, PKA, and CK-2 activities were examined at similar conditions except that myelin basic protein (a gift from Zvi Naor), CREB (Table 1), and CK-2 peptide were used as substrates, respectively. Protein kinase C- δ was immunoprecipitated with a specific antibody (Santa Cruz Biotechnology, Inc., Santa

Cruz, CA) from fat tissue extracts and its activity was measured at similar conditions except that the lipid cofactor phosphatidylserine (40 μ M) 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 Biolabs), and kinase assays were performed at similar conditions except that myelin basic protein 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-permeable L803 inhibitor L803-mts [*N*-Myristol-GKEAPPAP-PQS(p)P] and two similarly modified respective controls LE803-mts (*N*-Myristol-GKEAPPAPPQSEP) and LS803-mts (*N*-Myristol-GKEAPPAPPQSP), 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's medium supplemented with 10% fetal calf serum. On the day of the experiment, cells were incubated with low-glucose medium supplemented with 0.5% fetal calf serum for 1 h, 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 h. A vehicle control of DMSO (0.1%) 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 μ g/ml leupeptine, 10 μ g/ml aprotinine, 10 mg/ml pepstatin A, and 1 mM benzamide), scraped with the same buffer, and frozen in liquid nitrogen as described previously (Eldar-Finkelman et al., 1996). Glycogen synthase activity was assayed according to the method of Thomas et al. (1968) and based on the incorporation of uridine 5-diphosphate [¹⁴C]glucose into glycogen. Aliquots of cell lysates (15 μ l) were incubated with 15 μ l of reaction mixture [66.6 mM Tris, pH 7.8, 32.5 mM KF, 0.8 μ Ci/ μ l [¹⁴C]uridine 5-diphosphate [¹⁴C]glucose (400 μ M), and 13 mg/ml glycogen rabbit liver; Sigma-Aldrich] for 20 min at 30°C as described previously (Eldar-Finkelman et al., 1996). The reactions were then spotted on ET31 (Whatman, Maidstone, UK) papers, washed with 66% ice-cold ethanol, and counted for radioactivity. Glycogen synthase assays were measured in the presence of 0.1 mM glucose 6-phosphate. Similar results were obtained when glucose 6-phosphate 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 Biochemicals, Freehold, NJ), as described previously (Lawrence et al., 1977). Digested fat pads were passed through nylon mesh and cells were washed three times with Krebs-bicarbonate buffer (pH 7.4) containing 1% bovine serum albumin

TABLE 1

Peptide sequences used in these studies

The property of peptide as substrate or inhibitor (or neither) is indicated. S¹ and S² are marked bold in peptide substrate 1. Replacement of S¹ in other peptides is marked bold. S(p) represents phosphorylated serine.

Peptide	Length	Substrate	Inhibitor	Comments
1. KRREILS ¹ RRPS ² (p)YR	13	+	—	Derived from CREB
2. ILSRRPS(p)YR	9	+	—	p9CREB
3. ILSRRPEYR	9	—	—	
4. ILSRRPSYR	9	—	—	
5. KRREILARRPS(p)YR	13	—	+	Hz13
6. ILARRPS(p)YR	10	—	— ^a	
7. KEEPPSPQS(p)P	11	+	—	Derived from heat shock factor-1
8. KEEPPAPPQS(p)P	11	—	+	pAHSF
9. KEAPPAPPQS(p)P	11	—	+	L803
10. KEEPPAPPQSP	11	—	—	
11. KEEPPAPPQEP	11	—	—	
12. PAPPQS(p)P	7	—	— ^a	
13. EPPAPRRE	8	—	—	
14. EPPAPR	6	—	—	

^a Weak inhibition (IC₅₀ > 800 μ M).

(fraction V; Roche Diagnostics, 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 h, followed by addition of [3 H]2-deoxyglucose (0.5 μ Ci/vial) for 10 min. The assay was terminated by centrifugation of cells through dinonylphthalate (ICN Pharmaceuticals, Costa Mesa, CA), and 3 H was quantitated by liquid scintillation analyzer (PerkinElmer Life Sciences, Boston, MA). Nonspecific uptake of [3 H] 2-deoxyglucose was determined by the addition of cytochalasin B (50 μ M) 30 min before the addition of radioactive material. In another set of experiments, adipocytes were treated with various concentrations of L803-mts 1 h before addition of suboptimal concentration of insulin (5 nM). Glucose uptake was determined as described above.

In Vivo Studies

High-Fat Diet-Induced Diabetes in Animals. Four-week-old C57BL/6J mice received high-fat diet containing 35% lard (Bioserve, Frenchtown, NJ) with 55% of calories from fat as described previously (Surwit et al., 1988). Animals were housed in individual cages with free access to water in a temperature-controlled facility with 12-h light/dark cycle. Animals developed obesity, hyperglycemia, and hyperinsulinemia after 16 weeks of diet feeding (I. Talior, M. Yarkoni, N. Bashan, H. Eldar-Finkelman, manuscript submitted for publication). Studies were done according to the 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 h). L803-mts and LE803-mts were administered i.p. to mice (400 nmol of peptide) for 1 h, glucose (1 g/kg) was injected 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 Diagnostics). Similar experiments were performed in diabetic C57BL/6J mice that were fed high-fat diets for 16 weeks, except that mice fasted for 6 h, and L803-mts was injected 90 min before glucose injection. The stability of L803-mts in serum was tested. L803-mts (1 mM) was incubated with mouse serum (100 or 200 μ l) for 2.5 h in 37°C. Primary adipocytes were incubated with the "serum-treated" L803-mts, L803-mts (10 μ M in 10 μ l each), or serum alone (10 μ l), and glucose uptake was measured as described in the previous section. Results indicated that the serum-treated L803-mts exhibited 88% activity of the maximal activity achieved with the "nontreated" L803-mts.

Results

In Vitro Studies. The in vitro analyses were performed with purified GSK-3 β . Table 1 lists the peptides used in the present studies. Replacement of S¹ with alanine in two known peptide sequences derived from GSK-3 substrates cAMP-responsive element binding protein (CREB) and heat shock factor-1 (HSF-1) converted the peptides into inhibitors (Table 1, peptides 5 and 8). Replacement of the glutamic acid located upstream to S¹ in pAHSF peptide improved the potency of inhibition (L803; Table 1, peptide 9).

Figure 1, A and B, presents the inhibition curves of the three peptide inhibitors termed Hz13, pAHSF, and L803 and indicates their IC₅₀ values (range, 150–330 μ M). The kinetic nature of the peptide inhibitors was studied by measuring the initial velocity as a function of the substrate phosphorylation 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

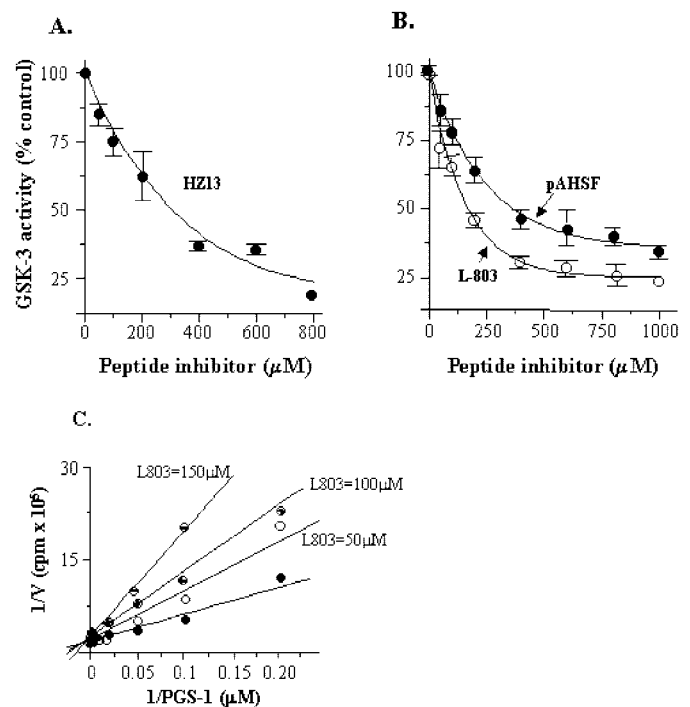


Fig. 1. In vitro kinetic analysis of phosphorylated peptide inhibitors. The ability of GSK-3 to phosphorylate PGS-1 peptide substrate was measured in the presence of indicated concentrations of peptides HZ13 (A), pAHSF and L803 (B). Results represent the percentage of GSK-3 activity in control incubation in which peptide inhibitors were omitted. Results are mean of three independent experiments \pm S.E.M., where each point was assayed in triplicate. IC₅₀ values are HZ13 = 330 μ M, pAHSF = 300 μ M, and L803 = 150 μ M. C, Lineweaver-Burk plot of inhibition of GSK-3 by L803 at indicated concentrations. Results represent phosphate incorporation into PGS-1 peptide substrate (counts per minute). Results show one representative experiment of four. Each point is a mean of duplicated sample. Calculated $K_i = 70 \pm 10$ μ M.

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 and 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 L803 peptide due to replacement of glutamic acid positioned upstream to S¹ 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 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 (200 μ M) to significantly inhibit a selection of

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 under *Materials and Methods*. Results present the percentage of the activity measured in the absence of the inhibitor.

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

protein kinases. Notably, the most closely related protein kinase to GSK-3, 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 used the myristoylated peptide L803-mts and two similarly modified respective controls LE803-mts and LS803-mts (see *Materials and Methods*). In vitro kinetic analysis indicated that L803-mts inhibited purified GSK-3 β ($\text{IC}_{50} = 40 \mu\text{M}$; Fig. 2A) and behaved as a competitive inhibitor (Fig. 2B). Interestingly, L803-mts was a better inhibitor than 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 h (Fig. 3A) indicated that L803-mts increased glycogen synthase activity by 2.5-fold compared with 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). Together, L803-mts (10–40 μM) 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 h before measuring the uptake of [^3H]2-deoxyglucose. As shown in Fig. 4A, L803-mts increased the incorporation of 2-deoxyglucose by approximately 2.5-fold compared with cells treated

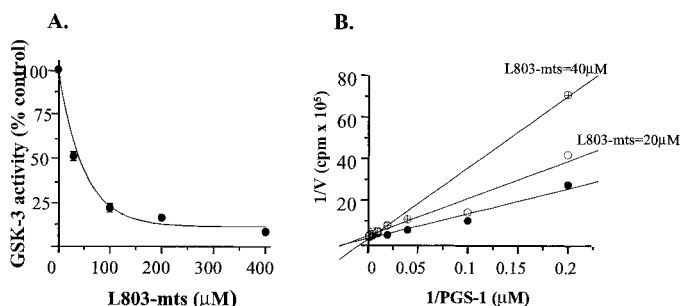


Fig. 2. In vitro kinetic analysis of L803-mts. A, ability of GSK-3 to phosphorylate PGS-1 peptide substrate was measured in the presence of indicated concentrations of L803-mts. Results represent the percentage of GSK-3 activity in control incubation in which peptide inhibitors were omitted. Results are mean of two independent experiments \pm S.E.M., 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 (counts per minute). Results show one representative experiment of three. Each point is a mean of duplicated sample.

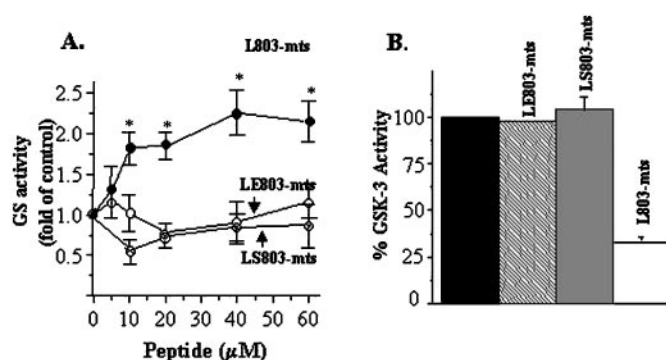


Fig. 3. L803-mts is a potent GSK-3 inhibitor in intact cells. HEK 293 cells were treated with L803-mts and control peptide LE803-mts or LS803-mts for 2.5 h at indicated concentrations. Lysate supernatants were assayed for glycogen synthase activity. A, activity of glycogen synthase in cells treated with vehicle DMSO (0.1%) 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 (LE803-mts, open circles; LS803-mts, circles with \times) are presented as fold stimulation over the cells treated with vehicle DMSO. Data are mean of three independent experiments \pm S.E.M. where each point was assayed in duplicate. *, value significantly greater than cells treated with control peptides. B, inhibition of purified GSK-3 β by L803-mts but not by control peptides LE803-mts or LS803-mts (50 μM each) is shown. In vitro kinase assays were performed as described in legend to Fig. 1. Black bar, no treatment.

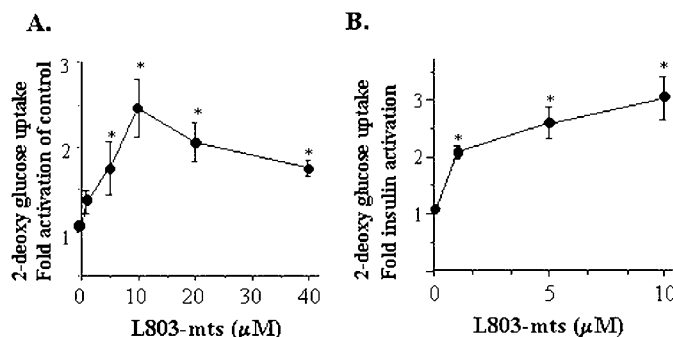


Fig. 4. GSK-3 peptide inhibitor increases glucose uptake in the absence or presence of insulin. A, adipocytes were isolated from mouse epididymal fat tissue and incubated with L803-mts for 75 min. Glucose uptake into cells was assayed with [^3H]2-deoxyglucose, as described under *Materials and Methods*. The relative [^3H]2-deoxyglucose incorporation observed in adipocytes treated with LE803-mts was normalized to 1 unit, and the values obtained for [^3H]2-deoxyglucose in adipocytes treated with LE803-mts are presented as fold activation over cells treated with the peptide control and are the mean of five independent experiments \pm S.E.M., 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 (5 nM) was added for another hour. Glucose uptake into cells was assayed with [^3H]2-deoxyglucose 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 four experiments \pm S.E.M., where each point was assayed in triplicate. *, value significantly greater than cells treated with insulin.

with LE803-mts or LS803-mts. This value is comparable with that attained by maximum stimulation by insulin (10 nM), which is 3-fold in these mouse adipocytes (data not shown). To determine whether our peptide GSK-3 inhibitor can work in concert with insulin, adipocytes were first treated with varied concentrations of L803-mts (1–10 μM) followed by the addition of a suboptimal 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 h after 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 h 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). Figure 6 shows that when HF mice were pretreated with L803-mts, their performance on glucose tolerance tests significantly improved (34% reduction in the blood glucose peak), and blood glucose clearance was much faster to return to basal levels compared with the animals treated with the control peptide LE803-mts.

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, SXXXXS(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 toward GSK-3. Indeed, the GSK-3 peptide inhibitor L803-mts did not inhibit a selection of protein kinases, including protein kinase C, PKB, and cdc2 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 syn-

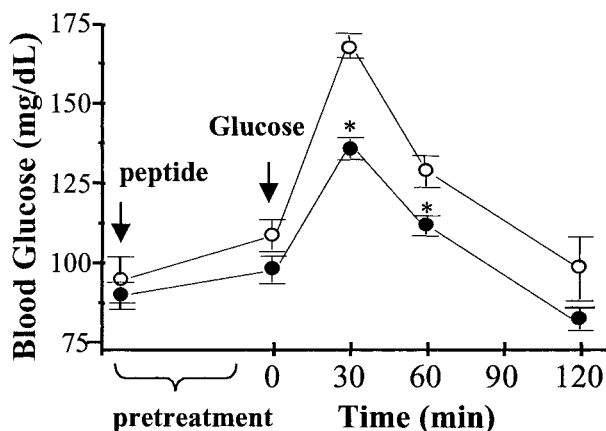


Fig. 5. GSK-3 inhibitor improves glucose tolerance. After overnight fasting, animals were injected i.p. with L803-mts or LE803-mts 1 h before injection of glucose. Blood glucose (milligrams per deciliter) levels were measured at the indicated time points. Results are mean of 12 animals treated with L803-mts (filled circles) or nine animals treated with control peptide LE803-mts (open circles) \pm S.E.M. *, value significantly less than animals treated with control peptide.

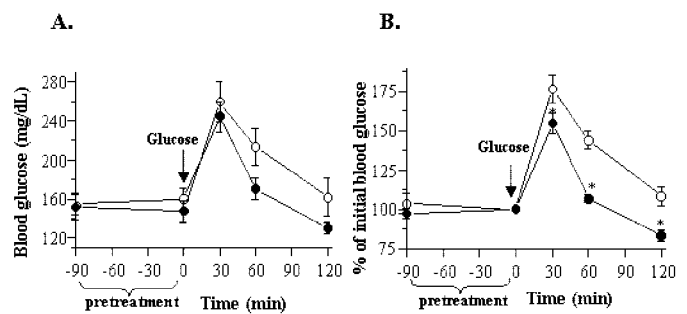


Fig. 6. GSK-3 inhibitor improves glucose tolerance in diabetic mice. A, after 6 h of fasting, HF mice were injected i.p. with L803-mts (filled circles) or LE803-mts (open circles) 90 min before injection of glucose (1 g/kg), and blood glucose levels were measured at the indicated time points. Results present the mean of 10 animals \pm S.E.M. B, results present the percentage of the blood glucose levels measured at the time of glucose injection (time 0). *, value significantly less than animals treated with control peptide.

these 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 suboptimal 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 insulin receptor substrate-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 antimicrobial agents of numerous invertebrates as well as certain vertebrates (Zaslhoff, 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; Lindsay, 2002; Tung and Weissleder, 2003) as well as the use of alternative delivery routes such as buccal 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 problems 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 bloodstream, and dramatically improved their in vivo pharmacokinetic 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 60 kDa (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 compared with their respective nonlipidated peptides (Wang et al., 2002). It is therefore suggested that L803-mts, which is conjugated to fatty acid, possess 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 *Materials 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 (Figs. 5 and 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 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 nonpeptide molecules targeting GSK-3.

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