Inhibition of glycogen synthase kinase-3 (GSK3) stimulates glycogen synthase and glucose transport by distinct mechanisms in 3T3-L1 adipocytes

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Running title: GSK3 modulates both glucose transport and glycogen synthase
Summary

The role of glycogen synthase kinase 3 (GSK3) in insulin-stimulated glucose transport and glycogen synthase activation was investigated in 3T3-L1 adipocytes. GSK3 protein was clearly present in adipocytes and found to be more abundant than in muscle and liver cell lines. The selective GSK3 inhibitor, LiCl, stimulated glucose transport and glycogen synthase activity (20% and 65%, respectively, of the maximal (1 µM) insulin response) and potentiated the responses to a submaximal concentration (1 nM) of insulin. LiCl- and insulin-stimulated glucose transport were abolished by the PI3-kinase inhibitor, wortmannin, however, LiCl stimulation of glycogen synthase was not. In contrast to the rapid stimulation of glucose transport by insulin, transport stimulated by LiCl increased gradually over 3-5 hours reaching 40% of the maximal insulin-stimulated level. Both LiCl- and insulin-stimulated glycogen synthase activity were maximal at 25 minutes. However, insulin-stimulated glycogen synthase activity returned to basal after 2 hours, coincident with reactivation of GSK3. After 2 hours exposure to insulin, glycogen synthase was refractory to restimulation with insulin, indicating selective desensitization of this pathway. However, LiCl could partially stimulate glycogen synthase in desensitized cells. Furthermore, coincubation with LiCl during the 2 hour exposure to insulin completely blocked desensitization of glycogen synthase activity. In summary, inhibition of GSK3 by LiCl: (1) stimulated glycogen synthase activity directly and independently of PI3-kinase, (2) stimulated glucose transport at a point upstream of PI3-kinase, (3) stimulated glycogen synthase activity in desensitized cells and (4) prevented desensitization of glycogen synthase due to chronic insulin treatment. These data are consistent with GSK3 playing a central role in the regulation of glycogen synthase activity and a contributing factor in the regulation of glucose transport in 3T3-L1 adipocytes.
Insulin stimulates glucose uptake, metabolism and storage in liver, muscle and adipose tissue. The binding of insulin to its receptor activates the receptor’s intrinsic tyrosine kinase leading to stimulation of phosphatidylinositol 3-kinase (PI3-kinase) and other downstream kinases such as protein kinase B (PKB/Akt), p70 S6 kinase and PKCζ (1). One target of PKB is the Ser/Thr kinase, glycogen synthase kinase 3 (GSK3) (2, 3). Two isoforms of GSK3, α and β, are broadly expressed and play multiple regulatory roles in development and metabolism (4). GSK3 is constitutively active in cells and is transiently inhibited following insulin treatment (3). Inactivation of GSK3 by insulin requires PI3-kinase and appears to be mediated by PKB phosphorylation of GSK3 on Ser21 (α) or Ser9 (β) (5). GSK3 plays an important role in the regulation of glycogen synthesis via inhibitory phosphorylation of glycogen synthase. Indeed, overexpression of GSK3β leads to inhibition of basal and insulin-stimulated glycogen synthase activity (6, 7). Insulin stimulation of glycogen synthesis is impaired in muscle from patients with Type 2 diabetes (8, 9). Although the mechanism of this impairment is not completely understood, altered kinetic behavior of glycogen synthase persists in cultured skeletal muscle cells from diabetic patients even after prolonged maintenance under normoglycemic and normoinsulinemic conditions (9). The observed changes in the kinetic behavior of glycogen synthase could be secondary to increased phosphorylation (9) and consistent with this, the level of GSK3 protein has been shown to be elevated in muscle of Type 2 diabetics (10).

A recent report suggests another potential role for GSK3 as a negative regulator of insulin signaling through phosphorylation of insulin receptor substrate-1 (IRS-1) (11). Phosphorylation of IRS-1 on Ser/Thr residues by GSK3 makes it a poorer substrate for the insulin receptor kinase,
leading to decreased phosphorylation on tyrosine in response to insulin stimulation (11). Ser/Thr phosphorylation of IRS-1 has been proposed as the mechanism whereby several agents, TNFα (12, 13), phorbol ester (14), or the phosphatase inhibitor okadaic acid (15), impair signaling through PI3-kinase and blunt insulin stimulation of metabolic responses. Thus, in addition to direct modulation of glycogen synthase, GSK3 may be one of several kinases that negatively modulate insulin signal transduction via phosphorylation of insulin receptor substrates.

A role for GSK3 as a negative regulator of IRS-1 tyrosine phosphorylation was proposed based on overexpression studies (11). It has not been established whether such a negative feedback loop exists in cells expressing physiological levels of GSK3. Therefore, the role of GSK3 in two insulin-regulated biological responses, glycogen synthase activation and glucose transport, has been examined in 3T3-L1 adipocytes using a GSK3 inhibitor, lithium ion (Li+) (16, 17). Li+ inhibits GSK3 in vitro with an IC50 of 2 mM but does not inhibit several other kinases such as protein kinase A, casein kinase II, MEK, ERK-1, SAPK, Raf or Trk (16, 17). In addition, in vivo studies in several organisms suggest that GSK3 is an important physiological target of Li+ action. For example, Li+ treatment of Xenopus embryos leads to a dorsalized phenotype similar to that observed in response to expression of a dominant negative mutant of GSK3 (16). In contrast, inositol monophosphatase (IMPase) inhibition, another action of Li+, fails to phenocopy the Li+ effect on development (16).

Li+ can be insulin mimetic, stimulating glycogen synthesis in adipocytes, muscle cells and hepatocytes (7, 18-21). Li+ also stimulates glucose transport in adipocytes and muscle cells (21-23). Stimulation of glucose transport in adipocytes is not due to IMPase inhibition (23), suggesting that this functional effect of Li+ could also be due to inhibition of GSK3. Li+ treatment can normalize insulin sensitivity in diabetic rats (24) and has been shown to increase
glucose tolerance in humans (25, 26) although not in all studies (27). The goal of the present work was to determine whether inhibition of GSK3 with Li+ exerts a positive influence on insulin signaling or alters insulin-induced desensitization of the signaling pathway in adipocytes.
Methods

Glucose Uptake: 3T3-L1 cells were obtained from ATCC and grown to confluence in 12-well tissue culture plates. Two days post-confluence, cell differentiation was initiated by incubation with 2 ug/ml insulin, 0.25 uM dexamethasone and 0.5 mM isobutyl-methyl-xanthine (IBMX) in standard medium (Dulbecco’s Modified Eagle Medium (DMEM, GibcoBRL) containing 10% fetal bovine serum and antibiotic-antimycotic) for 48 hours. Medium was removed and replaced with standard medium containing 2 ug/ml insulin. After 2 more days, cells were re-fed with standard medium. At a minimum of nine days post differentiation, adipocytes were washed twice with serum-free (SF) DMEM. Cells were then preincubated with SF-DMEM for 2.5 hours followed by aspiration of the medium and addition of 1 ml of SF-DMEM containing either insulin, lithium or insulin plus lithium at the indicated concentrations. At the indicated times, this medium was aspirated and one ml of uptake buffer (PBS, pH 7.4. containing 0.05 mM 2-deoxy-glucose (0.01 uCi/ml [U-14C]-2-deoxy glucose) was added per well. After 10 minutes at 37°C, cells were washed 3x with ice cold PBS and lysed by the addition of 0.5 ml PBS containing 1% Triton X-100. Uptake was quantitated by liquid scintillation.

Glycogen Synthase Activation: Adipocytes were washed and incubated in SF-DMEM as described above. SF-DMEM contained 0.25% BSA, except in studies using wortmannin since this inhibitor was inactive in the presence of BSA. Adipocytes were treated with insulin, lithium or insulin plus lithium as described in the figures, and then scraped into 600 ul of homogenization buffer (HB: 10 mM TRIS pH 7.4, 150 mM KF, 15 mM EDTA, 0.6 M sucrose, 1 mM PMSF, 1 mM benzamidine, 25 ug/ml leupeptin and 50 mM 2-mercaptoethanol). Cells were lysed by a hand-held polytron (10 s, Kinematica AG) and 10 ul of each lysate was assayed for glycogen synthase activity using the ‘high glucose-6-phosphate (G6P)/low G6P’ method (28).
in a final volume of 90 ul. Each sample was assayed in the presence of 10 mM G6P (the ‘high G6P’ assay) to determine total glycogen synthase activity, or 0.25 mM G6P (the ‘low G6P’ assay) in a buffer containing 50 mM TRIS pH 7.8, 25 mM KF, 12.5 mM EDTA, 7 mg/ml glycogen, and 200 uM UDP-[U-14C]-glucose (4.5 uCi/umol). Reactions were carried out at 30°C for 10 minutes and stopped by placing on ice and spotting 75 ul of reaction mix onto Whatman 31 ET Chroma filter paper. Glycogen was precipitated by washing filters in 50% ethanol for 5 minutes, followed by two more 1 hr washes in 50% ethanol, and a 5 minute wash in acetone. Filters were dried and ¹⁴C-glucose incorporation into glycogen was quantitated by liquid scintillation. Data were expressed as the ratio of glycogen synthase activity under conditions of ‘low’ and ‘high’ glucose-6-phosphate.

**GSK3 Assay:** Adipocytes were serum-deprived for 2.5 hrs in SF-DMEM containing 0.25% BSA. Cells were treated as indicated in the same medium and scraped into 400 ul of GSK3 homogenization buffer (50 mM TRIS pH 8.0, 50 mM β-glycerophosphate, 5 mM EGTA, 50 mM KF, 10 mM DTT, 1 uM microcystin, 1 mM PMSF, 1 mM Na₃VO₄, 1 mM benzamidine, 1x Calbiochem Protease Inhibitor Cocktail). Cells were homogenized with a 10 second burst at top speed with a hand-held polytron. Homogenates were centrifuged for 15 minutes at 15,000x g at 4°C. Supernatants were saved for GSK3 assay and protein concentrations were determined using the Bradford method with bovine γ-globulin as standards.

For the assay, 12.5 ul of extract (corresponding to ~10 µg protein) was mixed with 6.25 ul of 4x substrate and 6.25 ul of 4x ATP mix. The substrate, B-2B-(SP), is a biotinylated peptide corresponding to the single phosphorylation site of translation initiation factor 2B (eIF2B). This peptide (2B-SP: RRAAEELDSRAGpSPQL-OH) was reported to be selective for GSK3 and therefore useful for measuring GSK3 activity in cell extracts likely to contain other Ser/Thr
kinases (29). Non-specific kinase activity was determined in duplicate assays containing 200 mM LiCl which completely inhibits GSK3 activity in vitro, and was subtracted from incorporation in the absence of lithium. The concentration of biotinylated peptide in the assay was 0.6 mg/ml (326 uM). Reactions were initiated by addition of 4x ATP mix to yield final concentrations of 50 mM TRIS pH 7.4, 12.5 mM MgCl₂, 2 mM DTT, 100 uM ATP and 1 uCi of γ-labeled ³²P-ATP (3000 Ci/mmol; Amersham) resulting in a specific activity of ~880 cpm/pmol. Reactions were carried out at 30°C for 30 minutes and terminated by addition of 25 ul of 1 N HCl. Peptide was captured by applying 10 ul of the terminated reaction mix to SAM² Biotin Capture Membranes (Promega, Madison, WI). Membranes were washed according to manufacturer's instructions: once for 30 seconds in 2M NaCl, 3 times for 2 minutes each in 2M NaCl, 4 times for 2 minutes each in 2M NaCl, 15 mM phosphoric acid and twice for 30 seconds each in deionized water. Membranes were rinsed in 100% ethanol for 1 minute, dried at 60°C and peptide phosphorylation quantitated by scintillation counting. The resulting CPM were converted into milliunits (mU) of GSK3 activity in which 1 mU of GSK3 corresponds to 1 pmole/minute of ³²P incorporated.
Results

GSK3β was easily detectable by immunoblotting in differentiated 3T3-L1 adipocytes (Data not shown). The level of GSK3β in adipocytes was higher than that found in Fao hepatoma cells or L6 myocytes but lower than that found in the TE671 medulloblastoma cell line (Data not shown). GSK3 activity was detectable in as little as 500 ng of 3T3-L1 adipocyte cell lysate and increased linearly with increasing protein over a 10-fold range (Data not shown).

Therefore, we examined the effect of the GSK3 inhibitor, LiCl, on glucose transport in 3T3-L1 adipocytes (Figure 1A). LiCl dose-dependently stimulated glucose transport with a statistically significant increase being observed at 20 mM and above (Figure 1A). 50 mM LiCl increased transport two to three-fold over basal (mean: 2.8 fold, n = 13 expts, p < 0.0001 vs basal), which corresponds to approximately 19% of the response elicited by a maximal dose (1 µM) of insulin (Figure 1B). NaCl does not inhibit GSK3 (16) and at concentrations up to 50 mM did not stimulate transport (Figure 1A), suggesting the action of 50 mM LiCl was not due to non-specific salt effects. However, higher salt concentrations (100 mM, Figure 1A) appeared to act non-specifically since both LiCl and NaCl stimulated uptake. The relatively small stimulation of transport in response to LiCl alone contrasts with the potentiation observed when LiCl was combined with a submaximal dose (1 nM) of insulin (Figure 1B). LiCl at 1 mM, a concentration which did not stimulate transport by itself (Figure 1A), elicited a small but significant increase in transport induced by 1 nM insulin (11%, p = 0.015; Figure 1B). Higher LiCl concentrations were synergistic with 1 nM insulin, increasing transport 2-3 fold over that stimulated by 1 nM insulin alone (Figure 1B). Glucose transport induced by the combination of 1 nM insulin and 50 mM LiCl was 60% of that induced by a maximal insulin concentration rather than the 20% observed in response to 1 nM insulin alone. As previously reported (30), glucose transport in response to
insulin is inhibited by the PI3-kinase inhibitor wortmannin (100 nM; Figure 2). Transport stimulated by LiCl, whether in the presence or absence of 1 nM insulin, was also blocked by wortmannin (Figure 2), indicating that both agents require PI3-kinase for stimulation of glucose transport in adipocytes.

Glycogen synthase in 3T3-L1 adipocytes was activated in response to LiCl (Figure 3). Activation of glycogen synthase by 50 mM LiCl ranged from 43 to 103% of that in response to 100 nM insulin (mean: 61.4±8.2%, n = 7 expts), and the combination of LiCl and 1 nM insulin gave a supramaximal response (Figure 3A). NaCl (50 mM) did not stimulate glycogen synthase nor did it potentiate insulin stimulation of glycogen synthase (Figure 3A). In contrast to the results with glucose transport, LiCl-stimulated glycogen synthase activation was largely resistant to wortmannin inhibition, whereas that stimulated by insulin was not (Figure 3B). Thus, LiCl appears to be acting downstream of PI3-kinase to stimulate glycogen synthase.

Further distinctions between LiCl and insulin stimulation of glucose transport or glycogen synthase emerged from time course studies. Glucose transport was maximally elevated after 30 min of treatment with 1 μM insulin, and remained elevated during eight hours of insulin exposure (Figure 4). In contrast, treatment of adipocytes for 30 min with 50 mM LiCl induced a 2 to 3-fold elevation in glucose transport, which increased to approximately 5-fold over basal after 5 hours of incubation (Figure 4). Therefore, LiCl-stimulated glucose transport continued to increase over a more prolonged time course than that stimulated by insulin and maximum transport in response to 50 mM LiCl was only 33% of that in response to insulin. However, glucose transport in response to both agents remained elevated for the duration of an 8 hour exposure (Figure 4).
Glycogen synthase, in contrast, was maximally activated in response to either insulin (1 uM) or LiCl (50 mM) after 30 minutes of treatment (Figure 5). LiCl stimulated glycogen synthase activity at 30 min was 36% of that stimulated by insulin in this experiment (Figure 5). Glycogen synthase activity remained elevated in LiCl-treated cells during the 8 hours of exposure (Figure 5). However, glycogen synthase activation in insulin-treated adipocytes was transient, declining to basal levels by 2 hours and continuing to decline to below basal levels during 8 hours of insulin exposure (Figure 5). This transient activation of glycogen synthase by insulin is distinct from the persistent activation of glucose transport and suggests that the pathway to glycogen synthase is selectively desensitized. This contrasts with the persistent stimulation of glycogen synthase observed in response to LiCl (Figure 5).

Since GSK3 phosphorylates and inactivates glycogen synthase, and is transiently inactivated in response to insulin (2, 31), it was of interest to determine whether the reactivation of GSK3 following insulin treatment exhibited a temporal correlation with the observed inhibition of glycogen synthase in 3T3-L1 cells. Maximal inhibition of GSK3 activity in insulin-treated adipocytes was 48% and was observed at 25 min, coincident with the maximal activation of glycogen synthase (Figure 6). Reactivation of GSK3 occurred over the same time course as inhibition of glycogen synthase (Figure 6), indicating that glycogen synthase and GSK3 are reciprocally regulated in response to insulin stimulation in adipocytes.

In order to determine if the transient stimulation of glycogen synthase activity by insulin was due to a true desensitization of this pathway, adipocytes were incubated with or without insulin (1 uM) for 2 hours and then restimulated with insulin prior to measurement of glycogen synthase activity. Cells preincubated in the absence of chronic insulin exhibited 5 or 6-fold increases in glycogen synthase activity in response to acute insulin or LiCl, respectively (Figure 5).
In contrast, cells preincubated with insulin were completely resistant to restimulation with a second dose of insulin (1 uM; Figure 7). Glycogen synthase activity in cells restimulated with insulin was equivalent to that in cells that had been maintained continuously in insulin for the entire 3 hours (Figure 7). This indicates that the lack of insulin-stimulated glycogen synthase activity with prolonged treatment was not due to insulin degradation but to desensitization of insulin signaling to glycogen synthase. Interestingly, LiCl (50 mM) treatment of adipocytes preincubated with insulin stimulated glycogen synthase nearly 4-fold (Figure 7). Therefore, LiCl is able to bypass, at least partially, the step(s) in the insulin signaling pathway that are desensitized, consistent with its site of action being much more proximal to glycogen synthase.

To examine whether GSK3 might play a role in the insulin-induced desensitization of glycogen synthase activation, cells were incubated with insulin (1 µM) for 2 or 2.5 hours in the presence or absence of LiCl (50 mM) prior to measurement of glycogen synthase activity. Cells preincubated in the absence of insulin and then acutely stimulated with insulin for 25 minutes exhibited an approximate four-fold increase in glycogen synthase activity (Figure 8). In contrast, glycogen synthase activity in cells incubated with insulin for 2 or 2.5 hours was less than that observed in control cells (Figure 8). Incubation with LiCl (50 mM) alone led to a 2-fold increase in glycogen synthase activity (Figure 8). More importantly, preincubation with LiCl and inclusion of LiCl during the subsequent 2 hour incubation with 1 µM insulin completely blocked the desensitization of glycogen synthase by prolonged insulin treatment (Figure 8).
Discussion

Inhibition of GSK3 by LiCl in 3T3-L1 adipocytes stimulates both glycogen synthase and glucose transport. The data presented here suggest that GSK3 modulates glucose uptake and glycogen synthesis via distinct mechanisms, acting at points upstream and downstream of PI3-kinase, respectively, as diagrammed schematically in Figure 9. In the basal state, GSK3 is active and inhibits glycogen synthesis directly by phosphorylation of glycogen synthase and conversion to the inactive form (Figure 9A). Evidence presented here and discussed below suggests that GSK3 inhibits glucose transport indirectly by negatively modulating events upstream of PI3-kinase, possibly tyrosine phosphorylation of IRS-1 or IRS-2 (Figure 9A). The inhibition by wortmannin of glucose transport stimulated by LiCl and insulin suggests that PI3-kinase is required for the stimulatory action on glucose transport by both of these agents. The requirement for PI3-kinase activity in insulin-stimulated glucose transport is well documented (1). However, GSK3 is located downstream of PI3-kinase in the insulin signaling pathway (5). Therefore, a requirement for PI3-kinase for stimulation of glucose transport by GSK3 inhibition is consistent with a model whereby LiCl acts to relieve the negative modulation of upstream events by GSK3 (Figure 9B). The potentiation by LiCl of glucose transport stimulated by submaximal insulin (Figure 1B) is further evidence suggesting that GSK3 inhibition moderates an inhibitory influence on the insulin signaling pathway.

The onset of glucose transport in response to insulin is rapid, reaching a maximal value at the earliest time examined (Figure 4). In contrast, LiCl-stimulated transport increases gradually over 3-5 hours. Transport stimulated by LiCl alone is much less than that in response to a maximal dose of insulin. This slow, partial activation is suggestive of a gradual shift in the basal state of signaling to glucose transport due to the moderation of a tonic inhibitory factor. Taken
together, these data support the hypothesis that endogenous GSK3 is acting to inhibit insulin signaling via phosphorylation of upstream components, possibly IRS-1, as has been previously suggested (11). Notably, IRS-1 and IRS-2 tyrosine phosphorylation has not been observed to increase significantly in LiCl-treated adipocytes*, suggesting that very small changes in phosphorylation state may be sufficient to induce the increases in glucose transport seen in these studies. However, stimulation of PKB by a submaximal concentration of insulin was enhanced by LiCl treatment*, consistent with GSK3 modulation of upstream signaling events.

It was recently reported that glucose transport in 3T3-L1 adipocytes was stimulated by lithium to a similar extent as that observed in the present studies but with little or no effect on GLUT4 translocation (7). In contrast to results shown here (Figure 1B), Summers et al. (7) also stated that LiCl did not augment insulin-stimulated glucose uptake, although it was not clear whether a submaximal concentration of insulin was used. The wortmannin sensitivity of lithium-stimulated glucose uptake reported here (Figure 2) suggests PI3-kinase-dependent GLUT4 translocation as the mechanism of action, although a modification of preexisting plasma membrane glucose transporters cannot be excluded. Notably, data from Summers et al. show a trend, albeit insignificant, for increased GLUT4 translocation with increasing lithium concentration (7). Since lithium-stimulated glucose uptake was only a small fraction of the maximal insulin-stimulated uptake in both studies, the amount of GLUT4 translocation that would account for such a minor increase in transport may be below the limit of detection of the GLUT4 sheet assay. Alternatively, because LiCl-stimulated glucose transport continues to increase for 3-5 hours following addition (Figure 4), exposure to LiCl for longer than the 60 minutes used in the prior study may be required to see a significant increase in GLUT4 translocation.
Overexpression of a non-regulatable form of GSK3 (S9A-GSK3β) in 3T3-L1 cells decreased basal and insulin-stimulated glucose uptake by 30-40% at all insulin concentrations tested, without changing the EC$_{50}$ for insulin-stimulation (7). The relatively modest effect of GSK3 overexpression may be due to the fact that endogenous GSK3 is already active in the absence of insulin, providing a degree of negative tone. The increased GSK3 activity in transfected cells may provide additional inhibitory tone, consistent with a dampening of signaling to glucose transport at all insulin concentrations. Thus, one interpretation of all the data is that the basal level of transport can be reset in a negative or positive direction either by addition of more GSK3 activity or inhibition of GSK3 with LiCl, respectively. Taken together, the data support an inhibitory role for GSK3 in glucose transport at a site upstream of PI3-kinase, although the precise mechanism remains to be defined.

Selective inhibition of GSK3 by LiCl stimulates glycogen synthase more robustly than glucose transport, averaging 61% of the maximal insulin response, in a wortmannin-resistant manner. This is consistent with glycogen synthase being a direct substrate of GSK3, and with GSK3 playing a significant role in glycogen synthase regulation in adipocytes. Based on prior observations of low levels of GSK3 expression in 3T3-L1 adipocytes, it was suggested that GSK3 inhibition plays a secondary role to activation of protein phosphatase-1 (PP1) in insulin stimulation of glycogen synthase in these cells (32, 33). However, both GSK3β protein and enzyme activity were readily measured in the 3T3-L1 adipocytes employed in the present study. Furthermore, GSK3 inhibition and glycogen synthase activation in response to insulin appeared to be tightly coupled in 3T3-L1 adipocytes (Figure 6). Interestingly, LiCl plus a submaximal dose of insulin stimulated glycogen synthase to a greater extent than a maximal dose of insulin alone (Figure 3). This suggests that the residual GSK3 activity not inhibited by insulin was
sensitive to inhibition by LiCl, and that further reduction in GSK3 activity contributes to greater activation of glycogen synthase. This is also consistent with the tight coupling of GSK3 inhibition and glycogen synthase activation observed in these cells.

Insulin stimulation of glycogen synthase in 3T3-L1 cells was transient, with activity returning to basal after 2 hours of insulin treatment (Figs. 5, 6). After 3 hours in the presence of insulin, when GSK3 activity had returned to pre-treatment levels, the glycogen synthase activity ratio was significantly below basal levels. Subsequent addition of insulin was unable to stimulate glycogen synthase (Figure 7), indicating that insulin signaling to glycogen synthase had been desensitized. LiCl was able to bypass the block induced by pre-exposure to insulin and stimulate glycogen synthase, again suggesting that direct inhibition of GSK3 leads to glycogen synthase activation. Interestingly, when prolonged insulin treatment was carried out in the presence of LiCl, glycogen synthase activity remained elevated (Figure 8). This suggests that GSK3 activity is required for the inactivation and desensitization of glycogen synthase which occurs during prolonged insulin treatment. The mechanism of this desensitization is not clear, although the ability to bypass the block partially with LiCl suggests that insulin inhibition of GSK3 is blocked. Notably, expression of a membrane-bound, constitutively active form of PKB also appears to desensitize insulin signaling to glycogen synthesis while stimulating glucose transport (34), providing additional evidence that persistent activation of the insulin signaling pathway in adipocytes can selectively block signaling to glycogen synthesis.

In summary, inhibition of GSK3 by LiCl enhances insulin signaling to glucose transport and activates glycogen synthase in 3T3-L1 adipocytes. The effect of LiCl on glucose transport is upstream of PI3-kinase and is consistent with a model in which GSK3 plays a negative role in insulin signaling via phosphorylation of insulin receptor substrates (Figure 9). The activities of
glycogen synthase and GSK3 are reciprocally regulated in these cells, and exhibit tight temporal correlation, suggesting that GSK3 plays an important role in the regulation of glycogen synthesis. Selective desensitization of glycogen synthase activation was observed with prolonged insulin treatment and appears to require GSK3 activity. Since impairment of insulin-stimulated glycogen synthesis is a common feature of Type 2 Diabetes (35), further studies to understand its desensitization will be important to the development of new therapeutic strategies.
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Footnotes:

* (Orena and Garofalo, unpublished observations)
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Figure Legends:

Fig. 1. **Effect of LiCl on glucose uptake and potentiation of insulin-stimulated glucose uptake in 3T3-L1 adipocytes.** (A) Adipocytes were incubated for 1 hour with increasing concentrations of LiCl (open columns) or NaCl (filled columns), followed by 10 minutes in the presence 2-deoxy-glucose as described under “Experimental Procedures”. Data are expressed as the percent of basal glucose uptake in untreated cells. Each point is the mean of triplicate assays ± S.E. (B) Adipocytes were treated with 1 nM insulin alone or in combination with increasing concentrations of LiCl for 1 hour. Maximal uptake was determined by treatment with 1 µM insulin. Uptake was measured as described above. Columns represent the average of triplicate determinations ± S.E.

Fig. 2. **Inhibition of insulin and lithium-stimulated glucose uptake by wortmannin.** Adipocytes were treated for 25 minutes with insulin or lithium as indicated, in the absence (open columns) or presence (filled columns) of 100 nM wortmannin. Uptake was measured for 10 minutes as described under “Experimental Procedures”. Each column represents the mean of triplicate assays ± S.E.

Fig. 3. **LiCl stimulation of glycogen synthase activity in 3T3-L1 adipocytes is wortmannin resistant.** (A) Adipocytes were treated for 30 minutes with insulin at the indicated concentrations, or LiCl (50 mM) or NaCl (50 mM) in the absence or presence of 1 nM insulin. Glycogen synthase activity was assayed in cell lysates as described under “Experimental Procedures.” Data are expressed as the ratio of glycogen synthase activity measured under conditions of “low” (0.25 mM) G6P versus “high” (10 mM) G6P. (B) Adipocytes were treated
for 25 minutes with insulin at the indicated concentrations, or lithium (50 mM), in the absence (open columns) or presence (filled columns) of 100 nM wortmannin. Glycogen synthase activity was assayed as described above. Columns represent the mean of triplicate assays ± S.E.

Fig. 4. **Time course for insulin and lithium stimulation of glucose transport in 3T3-L1 adipocytes.** Adipocytes were incubated in SF-DMEM alone (open circles), 50 mM LiCl (closed circles), or 1 μM insulin (squares) for the indicated times after which glucose transport was measured for 10 minutes as described under “Experimental Procedures”. Each point represents the mean of triplicate values from 3-5 experiments ± S.E. Where error bars are not visible, they are smaller than the width of the symbols.

Fig. 5. **Time course for insulin and lithium stimulation of glycogen synthase in 3T3-L1 adipocytes.** Adipocytes were incubated in SF-DMEM alone (open circles), 50 mM LiCl (closed circles) or 1 μM (squares) for the indicated times after which cells were lysed and glycogen synthase activity was assayed as described under “Experimental Procedures”. Data are expressed as the ratio of activities measured under conditions of “low” G6P (0.25 mM) and “high” G6P (10 mM). Each point is the average of triplicate values ± S.E.

Fig. 6. **Time course for insulin regulation of glycogen synthase and GSK3 activities in 3T3-L1 adipocytes.** Adipocytes were treated with 1 μM insulin for the indicated times. Cell lysates were assayed for glycogen synthase (open circles) and GSK3 activity (closed circles) as described under “Experimental Procedures”. Each point represents the average of triplicate determinations ± S.E.
Fig. 7. **Prolonged insulin treatment desensitizes glycogen synthase to reactivation by insulin but not to lithium.** Adipocytes were preincubated in the absence (open columns) or presence (filled columns) of 1 µM insulin for 2 hours. Incubations (±insulin) were continued with fresh medium with or without 50 mM LiCl for an additional 1 hour as indicated. The column labeled +INS (3 hr) represents cells incubated in the same insulin-containing medium for the duration of the experiment. Cell lysates were assayed for GS activity and the activity ratios expressed as the mean of triplicate measurements ± S.E.

Fig. 8. **Lithium prevents insulin-induced desensitization of glycogen synthase activity.** Adipocytes were preincubated for 30 minutes in SF-DMEM alone (CTL) or in the presence of insulin (1 µM) or LiCl (50 mM) as indicated, followed by an additional 2 hours with no additions (CTL), 1 µM insulin (filled columns) or 50 mM LiCl ± 1 µM insulin as shown. For comparison to peak stimulation, the second column represents glycogen synthase activity in cells treated with insulin for 25 minutes [INS(25')]. Each column is the mean glycogen synthase activity ratio of triplicate assays ± S.E.

Fig. 9. **Proposed model for mechanism of regulation of glucose transport and glycogen synthase by GSK3.** (A) In the absence of insulin stimulation (Basal state) GSK3 is constitutively active, maintaining glycogen synthase (GS) in its phosphorylated and inactive state. Data reported here suggest that GSK3 also acts upstream of PI3-kinase (PI3K) to inhibit insulin signaling to glucose transport, although the precise site of action is not known. (B) Insulin stimulates phosphorylation of insulin receptor substrates 1 and 2 (IRS1/2) by the insulin
receptor kinase which leads to activation of PI3-kinase and downstream kinases, such as protein kinase B (PKB) and protein kinase C isoforms (PKC ζ/λ). PKB and/or PKC ζ/λ appear to be part of the pathway to stimulation of glucose transport, and PKB phosphorylates and inactivates GSK3. Inactivation of GSK3 leads to activation of glycogen synthase by accumulation of the dephosphorylated, active form. Lithium inhibition of GSK3 stimulates both glycogen synthase as well as glucose transport. Stimulation of transport by either lithium or insulin is sensitive to PI3-kinase inhibition (wortmannin). These data are consistent with two sites of action for GSK3, both upstream and downstream of PI3-kinase.
Figure A: GS Activity Ratio

LiCl: 
- -- -- 50 mM -- -- 50 mM

NaCl: 
- -- 50 mM -- -- 50 mM --

Insulin: 
- 100 nM -- 1 nM 1 nM 1 nM

Figure B: GS Activity Ratio

LiCl: 
- -- -- 50 mM -- -- 50 mM

Insulin: 
-- 1 μM -- 1 nM -- 1 nM

Legend:
- White bar: Control
- Black bar: Treatment
A. Basal State

IRS-1/2 → IRS-1/2-Y^p + PI3K → PKB
PKC ζ/λ → Glucose Transport → GS

GSK3

GS_{active} → GS_{PPP}^{inactive}
PP1 → Glycogen synthesis

B. Insulin- or Lithium-treated

Insulin

IRS-1/2 → IRS-1/2-Y^p + PI3K
PKB PKC ζ/λ → Glucose Transport → GS

Wortmannin

GSK3

GS_{active} → GS_{PPP}^{inactive}
PP1 → Glycogen synthesis

Lithium
Inhibition of glycogen synthase kinase-3 (GSK3) stimulates glycogen synthase and glucose transport by distinct mechanisms in 3T3-L1 adipocytes

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