Inhibition of Glycogen Synthase Kinase 3 Improves Insulin Action and Glucose Metabolism in Human Skeletal Muscle

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Glycogen synthase kinase (GSK)-3 has been implicated in the regulation of multiple cellular physiological processes in skeletal muscle. Selective cell-permeable reversible inhibitors (INHs) of GSK-3 (CT98014 and CHIR98023 [Chiron, Emeryville, CA] and LiCl) were used to evaluate the role of GSK-3 in controlling glucose metabolism. Acute treatment (30 min) of cultured human skeletal muscle cells with either INH resulted in a dose-dependent activation of glycogen synthase (GS) with a maximally effective concentration of ~2 μmol/l. The maximal acute effect of either INH on GS (103 ± 25% stimulation over basal) was greater than the maximal insulin response (48 ± 9%, P < 0.05 vs. INH); LiCl was as effective as insulin. The GSK-3 inhibitor effect, like that of insulin, was on the activation state (fractional velocity [FV]) of GS. Cotreatment of muscle cells with submaximal doses of INH and insulin resulted in an additive effect on GS FV (103 ± 10% stimulation, P < 0.05 vs. either agent alone). Glucose incorporation into glycogen was also acutely stimulated by INH. While prolonged (6–24 h) insulin exposure led to desensitization of GS, INH continued to activate GS FV for at least 24 h. Insulin and LiCl acutely activated glucose uptake, whereas INH stimulation of glucose uptake required more prolonged exposure, starting at 6 h and continuing to 24 h. Chronic (4-day) treatment with INH increased both basal (154 ± 32% of control) and insulin-stimulated (219 ± 74%) glucose uptake. Upregulation of uptake activity occurred without any change in total cellular GLUT1 or GLUT4 protein content. Yet the same chronic treatment resulted in a 65 ± 6% decrease in GSK-3 protein and a parallel decrease (61 ± 11%) in GSK-3 total activity. Together with the INH-induced increase in insulin-stimulated glucose uptake, there was an ~3.5-fold increase (P < 0.05) in insulin receptor substrate (IRS)-1 protein abundance. Despite upregulation of IRS-1, maximal insulin stimulation of Akt phosphorylation was unaltered by INH treatment. The results suggest that selective inhibition of GSK-3 has an impact on both GS and glucose uptake, including effects on insulin action, using mechanisms that differ from and are additive to those of insulin. Diabetes 51:2190–2198, 2002

Glycogen synthase kinase (GSK)-3 is a serine/threonine kinase originally discovered because of its ability to phosphorylate and inhibit glycogen synthase (GS) (1). Human GSK-3 exists as two isoforms, α and β, encoded by two distinct genes, located on chromosomes 19q13.1-2 and 3q13.3-q21, respectively (2). GSK-3 is constitutively active in resting cells and is inhibited by several hormones such as insulin, endothelial growth factor, and platelet-derived growth factor. Insulin has been demonstrated to cause inactivation of GSK-3, both in vivo (3,4) and in several cell types (5–7). The mechanism of inactivation is associated with phosphorylation of the specific serine residues Ser21 and Ser9 in GSK-3 isoforms α and β, respectively. Insulin deactivation of GSK-3 is phosphatidylinositol (PI) 3-kinase–dependent and probably occurs through protein kinase B/Akt (8,9), properties shared with insulin stimulation of GS.

GS activity is regulated by allosteric and covalent (phosphorylation/dephosphorylation) mechanisms (10–12). Whereas a number of kinases and phosphatases can act on GS, GSK-3 plays an important role (13–15). GSK-3 phosphorylates GS on three specific residues. This phosphorylation causes deactivation of GS and decreases its affinity to allosteric activation by glucose-6-phosphate. In addition, it has been reported that GSK-3 can also phosphorylate insulin receptor substrate (IRS)-1, a key early molecule in insulin-signaling cascades (16), suggesting the potential for involvement of GSK-3 in multiple stages of insulin action.

Lithium ion (Li) has been found to cause relatively specific inhibition of GSK-3 (15,17) and has been reported to have insulin-like effects on glucose metabolism, including increases in glucose uptake, activation of GS activity, and stimulation of glycogen synthesis in skin, muscle, and fat cells. However, Li can also inhibit myo-inositol-1-monophosphatase (18), 1,6 bisphosphatase, and other enzymes involved in regulation of glucose metabolism (19–21). Several other agents have also been reported to inhibit GSK-3 activity: Ro 31-8220, valproic acid, iodothbericin, staurosporin, sangivemycin, and hymenialdisine (22). Like lithium, some of these inhibitors have been reported to exhibit antidiabetic effects (valproate) or to
promote glycogen formation (iodotubercidin). However, these agents are not specific for GSK-3 inhibition and also inhibit other protein kinases involved in insulin signal transduction and/or glucose metabolism. Consequently, it has been difficult to confidently ascribe the insulin-like effects of these agents to GSK-3 inhibition alone.

Several recent observations suggest an important role for GSK-3 in the regulation of GS and possible involvement in the development of insulin resistance in type 2 diabetes. Overexpression of GSK-3 in 293 cells and C3T3-L1 adipocytes results in decreased GS affinity to glucose-6-phosphate as well as impaired insulin-stimulated IRS-1 phosphorylation (15,16). In addition, GSK-3 expression is elevated in skeletal muscle of type 2 diabetic subjects and is inversely related to whole-body insulin action (22). However, the physiological role of GSK-3 in insulin-sensitive tissues, such as skeletal muscle and especially its role in regulation of glucose metabolism, remains to be determined.

The current experiments used novel, potent, and selective inhibitors of GSK-3 to study the regulation of glucose metabolism and insulin action in the major insulin target tissue—skeletal muscle. These studies were performed in cultured human skeletal muscle cells, a system that closely reflects the in vivo metabolic behavior of skeletal muscle, including impaired insulin activation of GS (23–25).

**RESEARCH DESIGN AND METHODS**

**Materials.** UDP[14C]glucose, [3H]-2-deoxyglucose were obtained from DuPont NEN (Boston, MA). [32P]ATP was purchased from ICN Pharmaceuticals (Costa Mesa, CA). Horseradish-conjugated anti-rabbit and anti-mouse IgG was obtained from Amersham (Arlington Heights, IL). SuperSignal Chemiluminescent Substrate was obtained from Pierce (Rockford, IL). BSA (Fraction V) and glucose-6-phosphate were purchased from Boehringer Mannheim (Indianapolis, IN). Protein assay reagents and electrophoresis chemicals were purchased from BioRad Laboratories (Hercules, CA). Glycogen, 2-deoxyglucose, pepstatin, leupeptin, phospho-methylsulfonyl fluoride (PMSF), and other reagents and chemicals were purchased from Sigma (St. Louis, MO). Anti-GSK-3/Shaggy protein kinase family (51/46-kDa) monoclonal IgG, purified GSK-3α- and β- from rabbit skeletal muscle, and anti-rat COOH-terminal IRS-1 rabbit polyclonal IgG were purchased from Upstate Biotechnology (Lake Placid, NY). Akt antibody, which detects total Akt1, Akt2, and Akt3 isoforms, was obtained from Cell Signaling Technologies (Beverly, MA). The antibody against the S473 phosphorylated form of Akt1, Akt2, and Akt3 isoforms was obtained from Cell Signaling Technologies (Beverly, MA). Antibodies against GLUT1 (polyclonal) and GLUT4 were obtained from Abcam (Cambridge, MA). Antibodies against GLUT1 (polyclonal) and GLUT4 were obtained from Abcam (Cambridge, MA).

**Cell culture.** Skeletal muscle cell cultures were established from muscle tissue obtained by needle biopsy samples of the vastus lateralis. Muscle biopsy samples were obtained from 47 nondiabetic and type 2 diabetic patients. All subjects underwent a 2-h 75-g oral glucose tolerance test. Normal glucose tolerance was defined as a fasting glucose level <7.0 mmol/l and 2-h glucose level <7.8 mmol/l (27). The experimental protocol was approved by the Committee on Human Investigation of the University of California, San Diego. Informed written consent was obtained from all subjects after explanation of the protocol.

The method of culturing skeletal muscle cells from biopsy samples has been described in detail in several publications (24,25). Satellite cells were obtained by trypsin digestion of muscle biopsy material. Cells were propagated in culture by modifications of the methods described by Blau and Webster (28) and Sarabia et al. (29). When cells attained ~80% confluency, they were transferred to differentiation media. Differentiation into multinucleated myotubes was completed within 5–7 days. Media were changed every 48 h. When cells were treated chronically for 4 days, media with fresh agents were changed every other day. Acutely, cells were treated with the selective GSK-3 inhibitor CHIR 98024 or its IA in serum-free media for 30 min or as indicated, and in some cases, together with insulin (33 mmol/l) at the completion of the differentiation period.

**GS activity.** GS activity was measured as described in detail previously (25,30). Cells from six-well plates were washed with PBS and scrapped into 0.3 ml of extraction buffer (50 mmol/l HEPES, 10 mmol/l EDTA, 100 mmol/l NaF, 5 mmol/l diithiothreitol [DTT], 1 mmol/l leupeptin, 1 mmol/l pepstatin, and 200 μmol/l PMSF, pH 7.5). Protein concentration was determined by the Bradford method (31) with BSA as a standard. GS activity was determined at a physiological concentration of substrate (0.3 mmol/l UDP glucose) and expressed in nanomoles of UDP glucose incorporated into glycogen per minute per milligram of total protein and as fractional velocity (FV) (the ratio of GS activity at 0.1 mmol/l glucose to that at 1 mmol/l glucose divided by the activity at 10 mmol/l glucose-6-phosphate). This is believed to be an indicator of changes in the phosphorylation state of GS in response to insulin (12).

**Glycogen synthesis.** Glycogen synthesis was determined in differentiated myotubes as [14C]glucose incorporation into glycogen during a 1-h incubation at 37°C (24). After incubation, cells were rinsed four times with PBS at 4°C and solubilized in 1N NaOH at 55°C for 1 h. An aliquot (100 μl) of the lysate was removed for protein analysis. The final glycogen pellets were resuspended in 0.5 ml H2O, mixed with scintillation fluid, and radioactivity determined by liquid scintillation counting. Absolute results are expressed as nanomoles glucose converted to glycogen per milligram protein per hour.

**Assay of GSK-3 activity.** Total GSK-3 (α and β) activity was measured in the homogenates against a peptide substrate (RRAEELDAGpSPQLG) derived from the e-subunit of eIF2-SP. This peptide and control peptide [where the Ser(β) is replaced by Ala] were obtained from Chiron. After treatment, cells were rapidly washed with PBS at 4°C and then lysed in buffer containing 20 mmol/l Tris-HCl, 145 mmol/l NaCl, 10% glycerol, 5 mmol/l DTT, 1% Triton X-100, 0.5% NP-40, 200 μmol/l sodium orthovanadate, 200 μmol/l PMSF, 1 mmol/l leupeptin, 1 mmol/l pepstatin, and 10 mg/ml aprotinin, pH 7.5. Kinase assays were performed on the cell homogenates in reaction mixtures that included 3 mg/ml peptide (substrate or control), 110 μmol/l [γ-32P]ATP, 16 mmol/l MgCl2, 5% serum-free chlo-4, 1 mM BSA, 500 μmol/l NaF, 5 mmol/l Na3VO4, 200 μmol/l Tris, pH 7.4. Reactions were stopped by adding 0.03-ml aliquots on Whatmann P81 paper squares and immediately transferring to cold 100 mmol/l phosphoric acid. Squares were washed three times with 100 mmol/l phosphoric acid and once with acetone and counted in a liquid scintillation counter.

**Glucose uptake assay.** Glucose uptake measurements were performed as described previously (32). Serum-free media were added to the cells together with insulin (0–33 mmol/l), and the cells incubated for 120 min in a 5% CO2 incubator before washing, uptake assay, and solubilization. An aliquot of the suspension was removed for protein analysis using the Bradford method. The uptake of [14C]glucose was used to correct each sample for the contribution of diffusion. This procedure measures both the transport and phosphorylation of glucose. Control studies indicate that transport was the rate-limiting process under the assay conditions used.

**Immunoblotting.** On the day of the experiment, control and insulin- or INH-treated cells were washed three times with ice-cold PBS and homogenized in a buffer containing 20 mmol/l Tris-HCl (pH 7.5), 1.5 mmol/l EGTA, 1 mmol/l EDTA, 10 mmol/l NaF, 1 mmol/l DTT, 0.5 mmol/l sodium orthovanadate, 10 μg/ml aprotinin, 20 μg/ml leupeptin, 0.2 mmol/l PMSF, and 0.5% Triton X-100. The levels of GS-3 isoforms (α and β, Shaggy protein kinase family) were determined by Western blotting using monoclonal antibodies raised against a peptide with a common sequence for α and β GSK-3 isoforms. Partially purified rabbit GSK-3α- and GSK-3β- were included on each gel as internal controls. Other antibodies are described in RESEARCH DESIGN AND METHODS. Western blotting was performed by the method of Burnette (33). After SDS-PAGE (7.5 or 10%), proteins were transferred to nitrocellulose membranes. Nonspecific binding was reduced by incubation in a blocking solution containing Tris-buffered saline, 3% nonfat dry milk, and 0.05% Tween 20.
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RESULTS

Acute activation of GS by GSK-3 inhibitors. Figure 1 presents the structures of the GSK-3 inhibitor compounds used in the current study. Confirmation of GSK-3 inhibition is presented in Fig. 1B. Direct addition of CHIR 98023 to the GSK-3 assay reduced enzyme activity by 72 ± 7% (P < 0.0005), whereas a structurally related inactive compound (IA) had no effect (1 ± 17% inhibition). Detailed dose-response curves performed with purified kinases revealed inhibitory potencies greater than those seen with whole-cell extracts (26).

To determine whether specific inhibition of GSK-3 acutely controls GS activation in skeletal muscle, we used cell-permeable inhibitors of GSK-3—CHIR 98014 and CHIR 98023 (Chiron) (26), collectively referred to as INH and LiCl. Both INHs acutely activated GS FV in a dose-dependent manner with a similar concentration-response profile (CHIR 98023 shown in Fig. 2A). Maximal effects were observed at 1–2 μmol/l for both INHs. This effect was on the activation state (FV) of the enzyme because maximal GS activity was unaltered.

The effects of the inhibitors were compared with the maximal effects of insulin and LiCl (20 mmol/l). Stimulatory actions of the INHs were greater than the maximal insulin or LiCl effects (Fig. 2B). The structurally related compound that is inactive (IC50 100× >98014 or 98023) against GSK-3 (IA) had no effect on GS activity. Whereas values for GS FV (both basal and insulin-stimulated) in type 2 diabetic subjects were lower (P < 0.05) than those in the nondiabetic subjects, in agreement with earlier results (25), the relative effects of the INHs were similar in muscle cell cultures from nondiabetic and type 2 diabetic subjects (Fig. 2B). Because the absence or presence of diabetes did not influence the relative effects of INH treatment, data in the two subject groups were combined, although cells from each subject were assayed individually.

Possible interactions between GSK-3 inhibition and insulin action on GS were investigated by treating cells simultaneously with insulin and CHIR 98023. Cotreatment with submaximal concentrations of CHIR 98023 and insulin (10 mmol/l) had a synergistic effect on the GS activation state (Fig. 2C). Maximal insulin action was also augmented by CHIR 98023. CHIR 98023 addition after preincubation with a maximal insulin dose resulted in an amplification of the insulin effect on GS FV (data not shown) without altering total GS activity.

Kinetics of GS activation. Differences were observed between the time course of GS activation by insulin and GSK-3 inhibitors. Insulin rapidly activated GS FV (Fig. 3). Stimulation increased for up to 2 h, followed by a return to near-basal activity after 24 h of treatment (Fig. 3). Chronically insulin-treated cells were also desensitized to subsequent acute insulin exposure (not shown). Similarly, LiCl also rapidly increased GS FV (30 min), but no effect was seen after prolonged exposure (Fig. 3). A biphasic effect of CHIR 98023 on GS FV was observed. There was rapid activation (0.5–2 h) of a magnitude greater than or equal to that of insulin or LiCl, which continued to increase until 24 h of treatment and was sustained for 96 h. The maximal activated GS FV, approximately fivefold that of control activity, could not be stimulated further by addition of acute insulin (not shown).

Effect of GSK-3 inhibition on glucose uptake. GSK-3 has been proposed to play a role in insulin-regulated glucose uptake (34). Acute (2-h) exposure of muscle cells to a dose of CHIR 98023 that maximally activated GS FV resulted in an insignificant increase in glucose uptake (Fig. 4A) compared with the ~80% stimulation in response to insulin. Interestingly, similar to insulin, LiCl acutely stimulated glucose uptake (78 ± 16% increase, P < 0.05). As was the case for GS FV, prolonged exposure to insulin (24 h) or LiCl (96 h) led to a desensitization of glucose uptake, with both a return toward basal activity (Fig. 4A) and a failure to respond to subsequent acute insulin exposure (not shown). In contrast, INHs increased glucose uptake significantly at 6–24 h after CHIR 98023 treatment (284 ±
Moreover, the increase was sustained for up to 96 h of treatment (Fig. 4A). A similar response was seen with CHIR 98014 (Fig. 4B). The inactive compound had no effect on glucose uptake after either acute or prolonged (Fig. 4B) treatment. Although cells chronically treated with insulin did not respond to subsequent acute insulin treatment (not shown), INH-treated cells displayed a further response to insulin (Fig. 4B). Insulin-stimulated uptake after INH treatment was approximately double that seen in control cells, whereas it was normal in LiCl-treated cells. Thus, sustained exposure to CHIR 98023 and CHIR 98014 resulted in upregulation of both basal and insulin-stimulated glucose uptake in skeletal muscle cells.

Regulation of glycogen formation. The effects of GSK-3 inhibitors on the major fate of glucose in skeletal muscle—storage in glycogen—were also evaluated. Insulin and INHs acutely stimulated glucose incorporation into glycogen, whereas the inactive analog had no effect (Fig. 5). Similar results were observed in cultured liver cells using other selective inhibitors of GSK-3 (35). The responses to insulin and INHs were comparable. The combination of insulin and INHs tended \((P < 0.1)\) to stimulate glycogen formation greater than that seen with either agent alone, as might be expected from the effect of insulin on glucose uptake (Fig. 4A) combined with maximal GS activation due to INHs.

Effect of GSK-3 inhibitors on protein expression. Although the acute effects of INHs and LiCl on GS could be attributed to inhibition of GSK-3 activity, the temporally biphasic nature of the effect of CHIR 98023 on GS FV and glucose uptake suggests that additional mechanisms could be involved. Chronic (4-day) exposure of muscle cells to INHs resulted in a dose-dependent decrease \((65\%, P < 0.05)\) in the expression of GSK-3 protein (Fig. 6A). Both the \(\alpha\) and \(\beta\) isoforms were reduced \((P < 0.05\) vs. paired control; \(\dagger P < 0.05\) vs. paired insulin-treated value for same individual).
Neither the inactive compound nor LiCl had any effect. Under the same conditions, total GSK-3 activity was reduced by 61 ± 11% (P < 0.05). The decrease in enzyme activity is truly reflective of the decrease in GSK-3 protein and not a residual inhibitory effect of INH because cells were extensively washed before extraction and extracts were diluted during the assay, removing the effect of the inhibitors as might be expected, since the nature of the inhibition is ATP competitive and reversible (26). The results indicated that the effects of the INHs were on GS activity state because total GS activity was unaltered after inhibition is ATP competitive and reversible (26). The sustained (2- to 4-day) increase in glucose uptake could not be explained by an upregulation of glucose transporters because INH treatment had no effect (Fig. 6C; 100 ± 14% of control, n = 4) on total cell GLUT1 protein expression. In keeping with the lack of effect of chronic exposure on glucose uptake, neither the inactive analog (101 ± 2%) nor LiCl (87 ± 4%) influenced GLUT1 content. A similar lack of change was seen for total cellular GLUT4 protein expression (Fig. 6C) after either INH (96 ± 10% of control, n = 4) or inactive analog (98 ± 2%) treatment.

DISCUSSION
One approach to elucidating the importance of GSK-3 in insulin action and control of glucose metabolism has been the use of LiCl as an inhibitor of GSK-3. To better understand the role of GSK-3 in regulation of glucose metabolism, we used both LiCl and more selective and potent inhibitors of GSK-3. CHIR 98014 and CHIR 98023 have 500- to 10,000-fold selectivity for GSK-3 over a large number of tested kinases (26). For example, CHIR 98014 has a Kᵢ of ~10 nmol/l for GSK-3 (both isoforms with similar potency) and stimulates GS with an IC₅₀ of 50–100 nmol/l (26), orders of magnitude more selective than LiCl (17,34). In addition, both INHs (CHIR 98014 and CHIR 98023) appear to have higher affinities and better selectivity than several recently described GSK-3 inhibitors (35).
Besides stimulating GS, these compounds, collectively referred to here as INHs, can augment insulin action on glucose uptake into skeletal muscle from insulin-resistant Zucker diabetic fatty rats (36). The experimental system used in the current study is cultured human skeletal muscle cells, which maintain many of the metabolic properties of skeletal muscle, including impaired GS activity in cells from diabetic subjects (24,25).

As would be expected, INHs acutely activated GS in human muscle cells at concentrations far lower than LiCl. Other GSK-3 inhibitors have also been demonstrated to increase glycogen synthesis in liver cells (35). The currently studied INHs alone were more effective than either insulin or LiCl. The increase in the response to a submaximal insulin dose might indicate the ability of INHs to improve insulin sensitivity. The maximal phosphorylation and deactivation attainable in response to insulin is not enough to fully inactivate GSK-3, whereas INHs can directly and more completely block enzyme activity.

Prolonged treatment of muscle cells with INHs was tested as a model more closely resembling the therapeutic situation. In the overall time course of INH action on GS, we observed two phases: a rapid activation that was complete within a half hour and a gradual increase over 6–24 h that was sustained even longer. This was opposite to the response to insulin, with transient activation of GS and then development of desensitization, as has been reported by multiple investigators of other cell types (37).

The prolonged upregulation of GS activity in response to INH treatment involves GS activation because total GS activity and protein expression were unaltered. The progressive GS activation apparently involves downregulation of GSK-3 protein expression because total activity and protein expression were reduced to the same extent. Protein abundance of both isoforms, which are present at similar amounts in control cells, was decreased in INH-treated cells. The downregulation of GSK-3 proteins appears to involve inhibition of the enzyme because the response was observed with INHs and not the inactive analog. The lack of effect of LiCl on GSK-3 expression may be related to reduced potency over extended times and/or selectivity relative to INHs. Control of GSK-3 expression appears to be a second mechanism, available to INHs and not Li, for regulation of GSK-3 activity.

Several laboratories have reported that GS protein expression is normal in the skeletal muscle of type 2 diabetic subjects (30,38). Together with the evidence that GSK-3 expression is elevated in skeletal muscle of type 2 diabetic subjects (25), this would suggest that an abnormally low GS/GSK-3 ratio is a feature of diabetic skeletal muscle, shifting the balance toward inactivation of GS. This behavior is also present in cultured muscle cells, where the ratio of GS protein to GSK-3 in cells from diabetic subjects (0.52 ± 0.08, n = 7) is significantly lower than the same value in nondiabetic cells (1.09 ± 0.12, n = 7, P < 0.005). Agents that can both inhibit and downregulate GSK-3 may
have an added therapeutic benefit for downregulation of GSK-3, whereas not influencing GS expression would have the ultimate result of increasing the GS/GSK-3 ratio; such a change would drive GS and GSK-3 toward a more normal balance. The specific molecular mechanism(s) by which INHs downregulate GSK-3, impaired synthesis, and/or accelerated degradation are unknown and represent an intriguing area for further investigation.

Li has been shown in some (34,39,40) but not all (41,15) situations to acutely stimulate glucose transport or GLUT4 translocation. This response differs from activation of GS because it can be blocked with wortmannin (34), indicating that it occurs at the level of PI 3-kinase activation, whereas GS stimulation is not wortmannin sensitive and occurs downstream of PI 3-kinase (34,15). Cultured human skeletal muscle cells behave much like other cells because Li is as effective as insulin in acutely stimulating glucose uptake. Whether the glucose transport response to Li involves direct inhibition of GSK-3 is uncertain because INHs have little acute effect. Other mechanisms for Li action on glucose transport have been proposed after ruling out inhibition of myo-inositol-1-monophosphatase and accumulation of inositol monophosphates (40). These mechanisms may include effects on Ca2+ and 1,6 bisphosphate (19,41). In rat muscles, Li has been shown to change Ca2+ intracellular distribution, which could lead to activation of glucose transport (42). The lack of acute INH effect on glucose uptake in muscle cells is in agreement with studies in isolated muscles from ZDF rats (36), where uptake in the absence of insulin is unaltered by short-term exposure to CHIR 98014.

Insulin and Li have a similar activation profile on glucose uptake. These agents acutely stimulate glucose uptake followed by desensitization. In contrast, the INH effect on glucose uptake was delayed but caused a gradual and sustained increase. The increases in glucose uptake with INH treatment could not be attributed to changes in transporter protein expression, indicating that INHs may be acting on other aspects of transporter function, such as transporter intrinsic activity or localization to the plasma membrane; these questions remain to be studied. Unlike GS, where chronically activated enzymes in INH-treated cultures could not be stimulated further by insulin, glucose uptake could still be stimulated by insulin to a level approximately twofold greater than that possible in control cells. Although partly the result of an elevation of basal uptake, this final response suggests that insulin action may also be augmented.

The possibility that regulation of protein expression is one mechanism for the chronic effects of GSK-3 inhibitors is supported by the fact that GSK-3 phosphorylates and activates a number of transcription factors (43–45). GSK-3 is also a predominant kinase of translation initiation factor, eIF2B (46), phosphorylation of which causes its inactivation. Thus, inhibition of GSK-3 might reactivate protein synthesis at the level of translation. Alternatively, the upregulation of glucose uptake by INHs could be indirect—the result of a pull of intracellular glucose and glucose-6-phosphate into glycogen by activated GS.

There have been reports in which inhibition of GSK-3 has been shown to increase insulin action on glucose transport in isolated rodent skeletal muscles (26,41) and 3T3-L1 adipocytes (34,47). When seeking possible mechanisms for increased insulin action, we found that chronic INH treatment increases IRS-1 protein expression with a time course similar to that for INH effects on GS activity and glucose uptake. Such an upregulation of IRS-1 could potentiate insulin action, just as overexpression of IRS-1 has been shown to do in cell lines (48). The INH-induced upregulation of IRS-1 was not part of a generalized increase in insulin-signaling molecules because Akt expression was unaltered. IRS-1 has been identified as an in vivo substrate for GSK-3, and, acutely, GSK-3-mediated phosphorylation can impair its function (16). In addition, hyperphosphorylation of IRS-1 on serine residues induces IRS-1 degradation in proteosomes (49,50). Hence, inhibition and/or downregulation of GSK-3 could reduce IRS-1 phosphorylation and slow its degradation. Alternatively, IRS-1 protein synthesis could increase as a result of GSK-3 inhibition and reactivation of the translation initiation factor eIF2B (45). Whatever the mechanism, an increase in IRS-1 protein expression in skeletal muscle could augment insulin action, a further therapeutic benefit beyond activation of GS. This potential insulin-sensitizing action of GSK-3 inhibition might be even more obvious in vivo because we have recently demonstrated that muscle cells from type 2 diabetic subjects express normal amounts of IRS-1 protein (51), implicating something other than IRS-1 expression as the major site of impaired insulin signaling in vitro. Whatever the effects on insulin signaling, the INH-induced upregulation of IRS-1 expression was not found to increase insulin action on Akt phosphorylation. However, the current result was obtained at a maximally stimulating insulin concentration (33 nmol/l), which might obscure effects to improve insulin sensitivity.

The use of highly selective inhibitors has revealed several potential mechanisms by which GSK-3 can control glucose metabolism and insulin action in skeletal muscle. In the short term, GSK-3 acts to suppress GS activity and possibly reduce insulin sensitivity. In the long term, GSK-3 activity may provide a negative influence on glucose uptake and insulin signaling because inhibition of GSK-3 leads to upregulation of glucose uptake and insulin responsiveness. What is notable here is both the magnitude of upregulation with INHs and the delayed kinetics. In addition, GSK-3 activity appears to be required to sustain GSK-3 expression. Thus, along with expected effects on GS, GSK-3 can play roles in other more upstream events in insulin-signaling pathways. Sustained GSK-3 inhibition may represent a useful means of countering the reduced levels of muscle glucose uptake and metabolism characteristic in type 2 diabetes.

Whereas the current work has focused on the involvement of GSK-3 in glucose metabolism and insulin action, GSK-3 has been demonstrated to play a number of important roles, including control of cell differentiation and proliferation through the Wnt pathway (52) and regulation of specific gene transcription (53). Continuous activation of the Wnt pathway, associated with inhibition of GSK-3 activity, is often linked to tumor development (54). Additionally, a GSK-3β knockout in mice is embryonically lethal because of liver degeneration (55). Thus, inhibition of GSK-3 has the potential for broad effects beyond metabolism. Yet Li has an extended history of therapeutic
application, and augmented tumor formation has not been reported in those patients. In fact, evidence suggests that Li might even have a protective effect against cancer development (56). Considerable further study will be needed to sort out the various roles and regulation of GSK-3 in cell metabolism and development. Selective inhibitors of GSK-3 may prove to be useful tools in answering these questions.

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