Selective Glycogen Synthase Kinase 3 Inhibitors Potentiate Insulin Activation of Glucose Transport and Utilization In Vitro and In Vivo

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Insulin resistance plays a central role in the development of type 2 diabetes, but the precise defects in insulin action remain to be elucidated. Glycogen synthase kinase 3 (GSK-3) can negatively regulate several aspects of insulin signaling, and elevated levels of GSK-3 have been reported in skeletal muscle from diabetic rodents and humans. A limited amount of information is available regarding the utility of highly selective inhibitors of GSK-3 for the modification of insulin action under conditions of insulin resistance. In the present investigation, we describe novel substituted aminopyrimidine derivatives that inhibit human GSK-3 potently (Ki < 10 nmol/l) with at least 500-fold selectivity against 20 other protein kinases. These low molecular weight compounds activated glycogen synthase at ~100 nmol/l in cultured CHO cells transfected with the insulin receptor and in primary hepatocytes isolated from Sprague-Dawley rats, and at 500 nmol/l in isolated type 1 skeletal muscle of both lean Zucker and ZDF rats. It is interesting that these GSK-3 inhibitors enhanced insulin-stimulated glucose transport in type 1 skeletal muscle from the insulin-resistant ZDF rats but not from insulin-sensitive lean Zucker rats. Single oral or subcutaneous doses of the inhibitors (30–48 mg/kg) rapidly lowered blood glucose levels and improved glucose disposal after oral or intravenous glucose challenges in ZDF rats and db/db mice, without causing hypoglycemia or markedly elevating insulin. Collectively, our results suggest that these selective GSK-3 inhibitors may be useful as acute-acting therapeutics for the treatment of the insulin resistance of type 2 diabetes. Diabetes 52: 588–595, 2003

Type 2 diabetes is a leading cause of death in the developed world. This disease characteristically begins with insulin resistance in the peripheral tissues, and it is believed that potentiating insulin action may provide a valuable mode of treatment (reviewed in 1). After meals, insulin controls blood glucose levels by promoting glucose transport into peripheral tissues and enhancing formation of glycogen (2). At other times, glycogen formation in resting cells is suppressed via phosphorylation and inactivation of the rate-limiting enzyme glycogen synthase (GS) (3). Insulin indirectly relieves GS inhibition (4,5) through a signaling cascade beginning with phosphorylation of substrates, including insulin receptor substrate 1 (IRS-1), by the tyrosine kinase activity of activated insulin receptor (6,7). Tyrosine-phosphorylated IRS-1 initiates additional events, including inactivation of glycogen synthase kinase 3 (GSK-3; which is constitutively active in resting cells) and dephosphorylation of GS (7). Several enzymes have been implicated in the regulation of GS phosphorylation, including protein phosphatase 1G, cAMP-dependent protein kinase, casein kinase 1, and the highly homologous α and β isoforms of GSK-3 (1,8–10). There is convincing evidence that GSK-3 inactivation and GS activation are causally related, as GSK-3 phosphorylates GS at inactivating sites in vitro and overexpression of active forms of GSK-3 in cells suppresses GS function (11,12).

Both GSK-3α and GSK-3β are expressed in insulin-sensitive peripheral tissues (13,14), and abnormal overexpression of GSK-3 may contribute to the development of insulin resistance in rodents and humans. GSK-3 activity is elevated in obesity-prone diabetic rodents (15,16), and GSK-3 protein levels are significantly higher in muscle biopsies from patients with type 2 diabetes than in those from normal subjects (17). This elevation of GSK-3 correlates with the reduction in GS activity also seen in tissues from these patients with diabetes (17).

Additional support for a role of GSK-3 in the negative regulation of GS activity and insulin-dependent glucose transport arises from the discovery that lithium ions inhibit GSK-3 (18). Lithium salts have been reported to stimulate GS activity (12,13,19–23), increase glycogen deposition (12,19,22), and potentiate glucose transport activity (13,20,22,24,25) in a variety of cell types, and in

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Received for publication 30 April 2002 and accepted in revised form 20 November 2002.

vivo administration of lithium has been associated with anti diabetic effects (26–29). However, lithium is not an ideal reagent for investigating GSK-3, as high concentrations of the ion are needed to inhibit GSK-3 (5–10 mmol/l). Moreover, lithium inhibits other enzymes, including inositol monophosphatase and adenylyl cyclase (18), and are poorly tolerated in long term cell culture.

Recently, Coghlan et al. (23) reported activation of GS in cells with selective low molecular weight organic GSK-3 inhibitor. In rat hepatoma cells, these compounds cause a reduction in the expression of the gluconeogenic enzymes phosphoenolpyruvate carboxykinase and glucose-6-phosphatase (30). Potent and selective GSK-3 inhibitors, such as those reported by Coghlan et al. (23), will make it easier to define the role of GSK-3 in normal insulin signaling and in the development of insulin resistance and type 2 diabetes. The purpose of the present investigation was to describe the results of studies that have used a novel class of GSK-3 inhibitors, based on substituted aminopyrimidines, on GS activity in cell lines and isolated type 1 rat skeletal muscle, on glucose transport in type 1 skeletal muscle of the ZDF rat, and on whole-body glucose disposal in diabetic rodent models. With IC50 values as low as 1 mmol/l, these compounds are highly potent, and they show >500-fold selectivity for GSK-3 versus other kinase and non kinase enzymes. These GSK-3 inhibitors activate GS in cell lines and isolated muscle, enhance glucose transport in type 1 skeletal muscle of ZDF rats, and rapidly lower blood glucose levels when administered to ZDF rats or db/db mice.

RESEARCH DESIGN AND METHODS

GSK-3 inhibitors. GSK-3 inhibitors were >95% pure by high-performance liquid chromatography. Experiments in vitro used free base compounds diluted from DMSO stock solutions. Ex vivo and in vivo experiments used HCl salts formulated as described below.

Animals. Female db/db mice were obtained from The Jackson Laboratories (Bar Harbor, ME) at 6 weeks and used when 8–9 weeks of age. Male ZDF rats were obtained from Genetic Models Inc. (Indianapolis, IN) at 8–9 weeks and used at 10–13 weeks of age. Animals were fed Purina 5008 laboratory chow, received water ad libitum, and were maintained on a 12-h light/dark cycle (6:00 a.m., 6:00 p.m.) at 22–24°C.

Kinases and kinase assays. Erk2, protein kinase C (PKC)-ε, PKC-ζ, P90RSK2, c-src, AMPK, and pdk1 kinases were purchased from Upstate Biotechnology (Lake Placid, NY). DNA-PK was purchased from Cell Signaling Technology (Beverly, MA) at 25 units/ml and used at 20 units/ml each of DOPC and DOPS + 2 μmol/l PIPs); CHK1 (1.4 mmol/l, and 0.5 μmol/l biotin-cdc25 peptide); CK1 (3 mmol/l, and 0.2 μmol/l biotin-peptide); DNA PK (see 31); and phosphatidylinositol (PI) 3-kinase (5 mmol/l, and 2 μg/ml). Test compounds or controls were added in 5.5 μmol/l of DMSO, followed by 5 μl of AATP stock to yield a final concentration of 1 μmol/l ATP in all cell-free assays. After incubation, triplicate 100-μl aliquots were transferred to Cobiplate eight wells (LabSystems, Helsinki, Finland) containing 100 μl/well 50 μmol/l ATP and 20 mmol/l EDTA. After 1 h, the wells were rinsed five times with PBS, filled with 200 μl of scintillation fluid, sealed, left 30 min, and counted in a scintillation counter. All steps were performed at room temperature. Inhibition was calculated as 100% × (inhibited – no enzyme control)/(DMSO control – no enzyme control).

Enzyme and receptor panels. Selectivity against non kinase enzymes was tested on the Cerep “Enzyme” panel, including acetylcholinesterase; adenylyl cyclase; Na/K ATPase; cathepsin B and G; cytochrome c oxidase 1 and 2; EC2; epithelial growth factor receptor; elastase; guanylate cyclase; HIV-1 protease; inducible nitric oxide synthase; 5-lipoxygenase; monoamine oxidase A and B; phosphodiesterase I, II, III, and IV; PKC; phospholipase A2 and C; and tyrosine hydroxylase (Cell Line Europe, France). Selectivity against receptors was assessed on the MDS “Profiling the tyrosine receptor” panel, including adenosine (a1 and a2 nonselective and a1 and β1); cAMP; calcium channel type 1, M1, M2, and M3; dopamine D1 and D2; estrogen α; GABAA (agonist site and sodium channel); glucocorticoid; glutamate (NMDA/ reconnaissance and nonselective); glycine (glycine sensitive); histamine H1 (central); insulin; muscarinic M2 and M3; opiate δ, κ, and μ; phorbol ester; potassium channel; progestin (5-HT1 and 5-HT2/2selective); sigma (nonselective); sodium channel (site 2); and testosterone (MDS Pharma Services, Bothell, WA).

GS activity assays. CHO-IR cells expressing human insulin receptor, (provided by Hans Bock) were grown to 80% confluence in Ham’s F12 medium with 10% fetal bovine serum and without hypoxanthine (34). Trypanosed cells were seeded in 6-well plates at 1 × 10^6 cells/well in 2 ml of medium without fetal bovine serum. After 24 h, medium was replaced with 1 ml of serum-free medium containing GSK-3 inhibitor or control (final DMSO concentration <0.1%) for 30 min at 37°C. Cells were lysed by freeze/thaw in 50 mmol/l tris (pH 7.8) containing 1 mmol/l EDTA, 1 mmol/l DTT, 100 mmol/l NaF, 1 mmol/l phenylmethylsulfonyl fluoride, and 25 μg/ml leupeptin (buffer A) and centrifuged 15 min at 4°C/14000g. The activity ratio of GS was calculated as the GS activity in the absence of glucose-6-phosphate divided by the activity in the presence of 5 mmol/l glucose-6-phosphate, using the filter paper assay of Thomas et al. (35).

Primary hepatocytes from male Sprague Dawley rats that weighed ~140 g were prepared at the Rice Liver Laboratory (San Francisco, CA) and used 1–3 h after isolation. Aliquots of 1 × 10^6 cells in 1 ml of DMEM/F12 medium plus 0.2% BSA and GSK-3 inhibitors or controls were incubated in 12-well plates on a low-speed shaker for 30 min at 37°C in a CO2-enriched atmosphere, collected by centrifugation and lysed by freeze/thaw in buffer A plus 0.01% NP40; the GS assay was again performed using the method of Thomas et al. (35).

Isolated rat skeletal muscle incubations. Overnight-fasted animals were anesthetized with pentobarbital sodium (50 mg/kg i.p.). Soleus muscles were dissected into strips (~25 mg) and incubated for 1 h at 37°C in 3 ml of oxygenated (95% O2/5% CO2) Krebs-Henseleit buffer with 8 mmol/l glucose, 32 mmol/l mannitol, and 0.1% BSA (radioimmunoassay grade; Sigma Chemical) with or without the indicated concentrations of insulin (Humulin R; Eli Lilly, Indianapolis, IN) or the GSK-3 inhibitor. Thereafter, the muscle was used to assess the activity ratio (activity in the absence of glucose-6-phosphate divided by the activity in the presence of 5 mmol/l glucose-6-phosphate) of GS (35) or glucose transport activity, using 1 mmol/l 2-deoxyglucose (36).

Efficacy models. Blood was obtained by shallow tail snipping at lipodemean-inanesthetized tips. Blood glucose was measured directly (One-Touch Glucometer; LifeScan, San Jose, CA) or heparinized plasma was collected for measurement of glucose (Beckman Glucose Analyzer, Mountain View, CA) or insulin (Alpco Elisa, Windham, NH). Animals were prebled and randomized to vehicle control or GSK-3 inhibitor treatment groups. For glucose tolerance tests (GTTs), animals were fasted throughout the procedure with food removal early in the morning, 3 h before first prebleed (db/db mice), or the previous night, 16 h before the bleed (ZDF rats). When the time course of plasma glucose and insulin changes in fasting ZDF rats was measured, food was removed 18 h before the GTTs. After anesthetization, challenges in the GTT were 1.35 g/kg i.p. (ipGTT) or 2 g/kg via oral gavage (oGTT). Test inhibitors were formulated as solutions in 20 mmol/l citrate-buffered 15%
Captisol (Cydex, Overland Park, KS) or as fine suspensions in 0.5% carboxymethylcellulose.

Statistical analysis. The significance of differences between multiple groups was assessed by a factorial ANOVA with a post hoc Fisher’s protected least significant difference test (StatView version 5.0; SAS Institute Inc., Cary, NC). Differences between two groups were determined by an unpaired Student’s t test. \( P < 0.05 \) was considered to be statistically significant. All data are reported as means \( \pm \) SE.

RESULTS

CHIR 98014 and CHIR 99021 are highly selective inhibitors of GSK-3. A pool of substituted dihydropyrimidines that inhibited human GSK-3 at low micromolar concentrations was identified by screening of combinatorial libraries. The active compounds within the pool were found to be spontaneously oxidized aminopyrimidines with IC\( \text{50} \) for GSK-3\( \beta \) as low as 100 nmol/l. Further development of this series identified more potent compounds, including CHIR 98014 and CHIR 99021, which inhibited human GSK-3\( \beta \) with \( K_i \) values of 0.87 and 9.8 nmol/l, respectively (Fig. 1). These two compounds, as well as CHIR 99030 (a structurally similar compound), were also very effective in inhibiting murine and rat GSK-3, with IC\( \text{50} \) values in the low nanomolar range (Table 1).

Although both compounds acted as simple competitive inhibitors of ATP binding (data not shown), they exhibited from 500-fold to >10,000-fold selectivity for GSK-3 versus 20 other protein kinases (Table 1). Whereas CHIR 98014 and CHIR 99021 showed similar potency against the highly homologous \( \alpha \) and \( \beta \) isoforms of GSK-3, it is noteworthy that they strongly discriminated between GSK-3 and its closest homologs cdc2 and erk2. These three protein kinases all fall within the proline-directed serine/threonine kinase family and exhibit >30% amino acid identity within their catalytic domains. Several kinases that were tested are involved in the insulin signaling pathway (insulin receptor tyrosine kinase, akt1, pdk1, phosphatidylinositol [PI] 3-kinase, GSK-3). Among these, the GSK-3 isoforms

![FIG. 1. Inhibition of GSK-3 kinase activity by CHIR 98014 (A) and CHIR 98021 (B). Insets show chemical structures.](image)

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inhibitor CHIR 98014 activated the GS activity ratio in isolated type 1 skeletal muscle from insulin-sensitive lean Zucker and from insulin-resistant ZDF rats (Fig. 2C). Soleus muscle isolated from ZDF rats showed marked resistance to insulin for activation of GS but responded to 500 nmol/l CHIR 98014 to the same extent (40% increase) as muscle from lean Zucker rats. Notably, GS activation by insulin plus CHIR 98014 was additive in muscle from lean Zucker rats and greater than additive in muscle from the ZDF rats. Total GS activity was not altered by either CHIR 98014 or insulin in these cells and muscles (data not shown).

Selective GSK-3 inhibitors potentiate insulin-dependent glucose transport. Soleus muscle strips from lean Zucker or ZDF rats were incubated in vitro with increasing concentrations of insulin in the presence or absence of a maximally effective concentration of CHIR 98014 (500 nmol/l), and glucose transport activity was assessed. As expected, type 1 muscle from the ZDF rat was less sensitive to insulin than muscle from lean rats ($EC_{50}$ 115 vs. 59 nmol/l) and the maximal rate of glucose transport was reduced by half (Fig. 3). Whereas CHIR 98014 did not affect the insulin dose-response in muscle from lean animals (data not shown), it sensitized soleus muscle from ZDF rats to insulin, returning the $EC_{50}$ for insulin stimulation of glucose transport to 66 nmol/l and increasing the maximal rate of glucose transport to 71% of the rate in muscle from lean animals. Basal glucose transport was not affected in either group by the GSK-3 inhibitor.

Improved glucose disposal in diabetic rodents with GSK-3 inhibitor treatment. We next tested the effect of GSK-3 inhibitors on glucose disposal in several rodent models of type 2 diabetes. In ZDF rats, a single oral dose of CHIR 99021 rapidly lowered plasma glucose, with a maximal reduction of nearly 150 mg/dl 3–4 h after administration (Fig. 4A). Importantly, reduced fasting hyperglycemia was achieved while plasma insulin remained at or below control levels throughout the time course of the experiment (Fig. 4B). The response was reproducible and...
dose related (e.g., mild lowering at 8 mg/kg and maximal lowering at 30–48 mg/kg; data not shown).

The effect of in vivo administration of GSK-3 inhibitors on glucose tolerance was further assessed in oGTT and ipGTT. With the use of 10-week-old ZDF rats that display insulin resistance, glucose intolerance, and mild hyperglycemia, GSK-3 inhibitor CHIR 99021 was administered orally at 16 or 48 mg/kg 1 h before an oGTT. Animals in both treatment groups showed significantly improved glucose tolerance, with a 14% reduction in plasma glucose area under the curve (postchallenge) at 16 mg/kg inhibitor and a 33% reduction (*P < 0.05 vs. control) at 48 mg/kg (Fig. 5). It is noteworthy that in these diabetic rats, the higher dose of CHIR 99021 also reduced hyperglycemia before the oral glucose challenge. The improved glucose disposal in animals treated with the GSK-3 inhibitor occurred with no significant differences in plasma insulin levels compared with control animals.

Markedly diabetic and insulin-resistant db/db mice treated with 30 mg/kg CHIR 98014 also exhibited a significant reduction in fasting hyperglycemia within 4 h of treatment and showed improved glucose disposal during an ipGTT (Fig. 6). The response was dose dependent, as animals treated with 10 mg/kg showed a lesser response. Once again, improved glucose disposal in treated mice coincided with conservation or reduction of plasma insulin levels (Fig. 6, inset). As improved glucose tolerance was observed with either intraperitoneal or oral glucose challenges (Figs. 5 and 6), it seems unlikely that the
glucose-lowering mechanism was related to a compound effect on intestinal glucose delivery. The reduction in hyperglycemia and improved glucose disposal were not limited to db/db mice and ZDF rats, as similar results were observed with ob/ob mice, diet-induced diabetic C57BL/6 mice, and glucose-intolerant SHHF rats treated with CHIR 99021 or CHIR 98014 (data not shown).

DISCUSSION
We report in the present investigation on a new class of highly selective GSK-3 inhibitors that are effective at low nanomolar concentrations in enzyme assays and submicromolar concentrations in isolated cells and tissues. When tested against 20 protein kinases closely to distantly related to GSK-3, CHIR 98014 and CHIR 99021 showed >500-fold selectivity for GSK-3 (Table 1), and additional testing of CHIR 99021 showed >800-fold selectivity against 23 additional enzymes and 22 receptors. We have demonstrated that these compounds activate GS in cultured cells and in isolated type 1 diabetic rat skeletal muscle (Fig. 2) and enhance in vivo glucose disposal in rodent models of type 2 diabetes (Figs. 4–6).

Whereas similar effects caused by lithium have been ascribed to selective inhibition of GSK-3 (26–29), lithium inhibits other enzymes, including inositol monophosphatase and adenyl cyclase, at similar concentrations (18), leaving some uncertainty that the observed responses were due solely to GSK-3 inhibition. The GSK-3 inhibitors described in the present investigation are substantially more potent than lithium and even more potent than the GSK-3-selective maleimide compounds recently described by Coghlan et al. (23). We report here for the first time evidence that these selective GSK-3 inhibitors can rapidly lower blood glucose levels (fasting or after glucose challenge) in diabetic rodent models (Fig. 4) and can enhance glucose transport as well as GS activation in insulin-resistant oxidative skeletal muscle from type 2 diabetic rats. Within the aminopyrimidine series from which we selected CHIR 98014 and 99021, only GSK-3 inhibitors showed these properties, as close structural analogs that did not inhibit GSK-3 also failed to enhance GS activation or glucose disposal (D.B.R. and colleagues, unpublished data).

We expected the GSK-3 inhibitors in the present investigation to activate GS in tissues, because GSK-3 is known to phosphorylate and inhibit GS, GSK-3 is constitutively active in cells, and previous studies with lithium (13–16,19) and other synthetic GSK-3 inhibitors (23) have demonstrated GS activation. Considering the high selectivity of CHIR 98014 and 99021, our results argue even more strongly that inhibition of GSK-3 alone is sufficient to stimulate GS activity under many conditions. This does not preclude the possibility that GS is at times regulated by other mechanisms, in place of or in concert with GSK-3. Indeed, the contribution of insulin-stimulated effectors other than GSK-3 to modulation of GS activity may explain why we observed additivity or synergy between insulin and GSK-3 inhibitors in isolated rat skeletal muscle (Fig. 2). It has been proposed, for example, that most GS activation in adipocytes involves insulin stimulation of GS phosphatase protein phosphatase 1G (37), because platelet-derived growth factor partially inhibits GSK-3 in adipocytes without stimulating GS. However, these results could also be explained if platelet-derived growth factor inhibits only a subtraction of cellular GSK-3 that is not involved in GS regulation. The existence of such functionally distinct GSK-3 populations within the cell was proposed recently (38).

We observed that GSK-3 inhibition sensitizes soleus muscle to insulin, with an additive response of GS activation to insulin and GSK-3 inhibitor in normal muscle and more than additive enhancement in insulin-resistant soleus muscle from diabetic animals (Fig. 2). Furthermore, addition of GSK-3 inhibitor CHIR 98014 to soleus muscle from these diabetic rats also increased insulin-stimulated glucose transport, both by shifting the dose-response curve to the left and by raising the maximal response at maximally effective insulin concentrations (Fig. 3). In effect, the GSK-3 inhibitor partially reversed the glucose transport defects of diabetic muscle, generating an insulin response curve intermediate between those of diabetic and normal muscle. These results demonstrating a potentiation of in vitro insulin action on GS and glucose transport in rat muscle by selective GSK-3 inhibition are in agreement with the recent findings of Nikouli et al. (39), who showed in cultured human myocytes that these same GSK-3 inhibitors upregulate insulin-stimulated GS activity and glucose transport activity. A similar increase in response to insulin was seen by Tabata et al. (24) using the less selective agent lithium, although their results differed from ours in certain respects. They observed lithium-induced insulin sensitization in normal muscle, whereas we observed sensitization only in insulin-resistant muscle, and we did not see any stimulation of glucose transport by the GSK-3 inhibitor in the absence of insulin. The reasons for these differences are not clear, although they may involve effects of lithium on metabolic enzymes other than GSK-3.

It seems unlikely that the effect of GSK-3 inhibitors on glucose transport is a consequence of GS activation, because it has been demonstrated that the rate-limiting step in glucose uptake into muscle is entry into the cell and not deposition as glycogen (40). Indeed, we observed that activation of GS is not tightly correlated with glucose transport. Addition of CHIR 98014 to isolated soleus muscle from ZDF rats in the absence of insulin-stimulated GS activity without affecting glucose transport (Fig. 3). Furthermore, the GSK-3 inhibitors activated GS in normal liver and muscle but did not stimulate glucose transport or lower blood glucose in normal animals (Fig. 3 and data not shown). The in vitro activation of insulin-stimulated glucose transport in the soleus by GSK-3 inhibitors is also associated with enhanced GLUT-4 translocation (41). It is unlikely that this latter effect is a direct result of GS activation.

It is likely that events other than GS activation are responsible for the observed increase in glucose transport into insulin-treated diabetic muscle. GSK-3 has been shown to phosphorylate IRS-1 on serine residues (42), and it has been shown that serine phosphorylation of IRS-1 can interfere with insulin action (43,44). Together, these observations suggest that GSK-3 phosphorylation of IRS-1 could contribute to insulin resistance and that inhibition of GSK-3 could lead to an increase in insulin-dependent...
glucose transport independent of effects on GS activation. Consistent with the hypothesis that the effects of GSK-3 inhibition on glucose transport are not mediated by GS activation, the positive effect of lithium on glucose transport is sensitive to the PI 3-kinase inhibitor wortmannin, whereas lithium’s effect on GS is wortmannin-independent (12,13,22). Furthermore, divergence between GSK-3 effects on glucose transport and GS is consistent with our data demonstrating that GSK-3 inhibitors activate GS to a similar extent in normal and insulin-resistant muscle but activate glucose transport only in insulin-resistant muscle. Our observation that GSK-3 inhibitor administration in vivo reduces fasting hyperglycemia in ZDF rats (Fig. 4) suggests an ability of these compounds to modulate net hepatic glucose output. This is consistent with the recent findings of Cline et al. (45) demonstrating that GSK-3 inhibition with CHIR 99023 increased hepatic glycogen synthesis and decreased hepatic glucose output, and with Lochhead et al. (30) indicating that the selective reduction of GSK-3 activity with a different class of inhibitor (23) caused a diminution of the level of glucogenic enzymes phosphoenolpyruvate carboxykinase and glucose-6-phosphatase in rat hepatoma cells.

Concern has been expressed that long-term inhibition of GSK-3 may increase carcinogenic risk as a result of induction of β-catenin–regulated transcription factors. However, it should be noted that long-term use of the nonspecific GSK-3 inhibitor lithium is not known to be associated with increased risk of cancer. Moreover, regarding the wnt pathway, a 20-h infusion of CHIR 99021 in ZDF rats (which was equivalent to a daily dose of 130 mg/kg, more than threefold greater than the EC50 for glucose lowering in this animal model of type 2 diabetes [Fig. 4]), does not cause an induction of cytosolic β-catenin protein levels or cyclin D1 mRNA levels in brain, liver, lung, colon, or adipose tissues (D.B.R., unpublished data). Moreover, the GSK-3 inhibitor does not elevate β-catenin in normal cells, likely because the GSK-3 inhibition is not sufficient to stabilize β-catenin (S.D.H., unpublished data). This is in contrast to partially transformed cells, in which both our group (S.D.H., unpublished data) and Coghlan et al. (23) demonstrated an elevation of β-catenin with GSK-3 inhibitors, possibly as a result of PKC pathway activation. Moreover, unlike transformation with an activated ras oncogene, addition of the GSK-3 inhibitors to NIH3T3 and rat1 fibroblasts was not sufficient to allow cell growth in soft agar (S.D.H., unpublished data). Certainly longer-term treatments of cells and animal will be necessary to address more adequately this important issue.

In summary, our results demonstrate that these low molecular weight aminopyrimidine compounds are highly selective inhibitors of GSK-3 and function in the nanomolar range. Moreover, our results highlight the ability of these selective GSK-3 inhibitors to enhance insulin action in insulin-resistant skeletal muscle and improve glucose tolerance in rodent models of type 2 diabetes. These findings suggest that such compounds may potentially be therapeutically useful for treating diabetes and other insulin-resistant states, such as syndrome X, obesity, and polycystic ovary syndrome.

ACKNOWLEDGMENTS

This work was funded by Chiron Corporation.

We are very grateful to Jake Pritchett for hepatocyte isolation; to Mary Beth Giacona, Brenda Ho, Glenda Pollow, Caroline Low, Kate Fawcett, Christine Damico, and Brett Hensley for pharmacology assistance; to Melanie B. Schmidt and Mary K. Teachey for technical assistance with isolated muscle experiments; to Marion Wiesmann for comments on the manuscript; and to Veronica Martinez for administrative assistance. We also thank Lynn Seely, Fred Cohen, Gerald Shulman, Gary Cline, Robert Henry, Pete Peterson, and Walter Shaw for helpful discussions during the progress of this work. We are indebted to Rusty (Lewis T.) Williams for support and guidance from the outset.

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