Modulation of muscle insulin resistance by selective inhibition of GSK-3 in Zucker diabetic fatty rats


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ulated glucose transport in insulin-resistant skeletal muscle.

Recently, a class of novel and selective organic inhibitors of GSK-3 has been developed (27) and has shown promise as modulators of GS activity, glucose disposal, and glucose transport activity in diabetic rodent models (27) and in human muscle cells (21). These compounds are substituted aminopyrimidine molecules and act as potent competitive inhibitors (acting at the ATP-binding site) of human GSK-3 (Kᵢ < 10 nM) with ≥500-fold selectivity against 20 other protein kinases (27). The purposes of the present investigation were 1) to compare the in vitro effects of a novel, selective organic inhibitor of GSK-3 (CT-98014) and lithium ions on basal and insulin-stimulated GS activity and glucose transport activity in type I (soleus) and type IIb (epitrochlearis) skeletal muscle of insulin-sensitive lean Zucker (Fa⁻/⁻) and insulin-resistant Zucker diabetic fatty (ZDF) rats, the latter being a model of type 2 diabetes; 2) to assess whether any improvement of muscle glucose transport activity due to this GSK-3 inhibitor is associated with enhanced cell-surface GLUT4 protein; and 3) to determine the effect of the acute oral administration of a GSK-3 inhibitor (CT-98023) to ZDF rats on glucose tolerance and skeletal muscle glucose transport activity.

METHODS

Animals. Male ZDF/Gmi-fa rats were obtained from Genetic Models (Indianapolis, IN), and lean Zucker rats were purchased from Harlan (Indianapolis, IN) at the age of 8–9 wk and used in the experiments at 10 wk of age. At the time of their use, the ZDF rats weighed 300–340 g, whereas the age-matched lean Zucker rats weighed 180–210 g. Lean animals were maintained on regular lab chow (Purina, St. Louis, MO), whereas ZDF animals had free access to diabetogenic chow (Purina 5008). All procedures were approved by the University of Arizona Animal Use and Care Committee.

In vitro treatments of skeletal muscle. After an overnight fast, animals were deeply anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg), and intact epitrochlearis muscles and strips of soleus muscles (∼25 mg) were prepared for in vitro incubation in the unmounted state. Each muscle was incubated for 1 h at 37°C in 2 ml of oxygenated (95% O₂, 5% CO₂) Krebs-Henseleit buffer (KHB) with the NaHCO₃ concentration set at 14 mM. This KHB was supplemented with 8 mM glucose, 32 mM mannitol, 0.1% BSA (radioimmunoassay grade, Sigma Chemical, St. Louis, MO), 0.5% dimethyl sulfoxide, and the indicated additions of GSK-3 inhibitor, insulin, or lithium chloride. Thereafter, the muscles were used for the determination of glucose transport activity [as assessed using 2-deoxy-[1,2-3H]glucose (2-DG) uptake] or GS activity as described in Muscle glucose transport activity.

Oral administration of GSK-3 inhibitor. Animals were fasted after 6 PM of the evening before the test. At 8 AM and then again at 11:30 AM, the animals received by gavage either vehicle (1% carboxymethylcellulose/sodium/0.1% Tween) or a bolus of GSK-3 inhibitor CT-98023 at a dose of 30 mg/kg body wt. Thirty minutes after the second administration, some animals underwent an oral glucose tolerance test (OGTT). These animals received a 2 g/kg body wt glucose load by gavage. Blood was collected from a small cut at the tail immediately before and at 30, 60, 90, and 120 min after glucose administration, thoroughly mixed with EDTA (final concentration of 18 mg/ml), and centrifuged at 13,000 g to isolate the plasma. The plasma was stored at −80°C and subsequently assayed for glucose (Sigma Chemical) and insulin by radioimmunoassay (Linco, St. Louis, MO).

Other animals were used for the in vitro assessment of insulin-stimulated glucose transport activity in skeletal muscle. At the indicated times after the final bolus of vehicle or GSK-3 inhibitor, the animals were deeply anesthetized with pentobarbital sodium, and epitrochlearis muscles and soleus strips were prepared. All muscles were then incubated as described for 60 min in KHB in the absence or presence of the indicated concentration of insulin. Thereafter, glucose transport activity was assessed as described in Muscle glucose transport activity. At the time of muscle dissection, samples of plasma and soleus and plantaris muscles were taken and frozen in liquid nitrogen for the determination of GSK-3 inhibitor levels by high-performance liquid chromatography and mass spectrometry.

GS activity. GS was assayed essentially as described by Thomas et al. (35), with slight modifications (15). Muscles were homogenized in a Duall tube containing 2 ml of ice-cold 50 mM Tris buffer (pH 7.8), 100 mM KF, 10 mM EDTA, 2 mM EGTA, and 2 mM KH₂PO₄ at 4°C. The homogenates were centrifuged at 13,000 g for 15 min. An aliquot (30 μl) of the supernatant was added to 60 μl of 50 mM Tris buffer (pH 7.8), 20 mM EDTA, 25 mM KF, 10 mg/ml rabbit liver glycogen, and 5 mM UDP-[U-14C]glucose (150 μCi/mmol) and incubated for 20 min at 30°C without glucose 6-phosphate (GS₆ activity). Total GS activity was assessed in the presence of 5 mM glucose 6-phosphate.

Muscle glucose transport activity. After the initial incubation period, the muscles were rinsed for 10 min at 37°C in 3 ml of oxygenated KHB containing 40 mM/l mannitol, 0.1% BSA, and any addition present previously. Thereafter, the muscles were transferred to 2 ml of KHB containing 1 mM 2-deoxy-[1,2-3H]glucose (300 μCi/mmol; Sigma), 39 mM [U-14C]mannitol (0.8 μCi/mmol; ICN Radiochemicals, Irvine, CA), 0.1% BSA, and any additions present previously. At the end of this final 20-min incubation period at 37°C, the muscles were removed, trimmed of excess fat and connective tissue, quickly frozen between aluminum blocks cooled in liquid nitrogen, and weighed. The frozen muscles were dissolved in 0.5 ml of 0.5 N NaOH. After the muscles were completely solubilized, 5 ml of scintillation cocktail was added, and the specific intracellular accumulation of 2-DG was determined as described previously (14). This method for assessing glucose transport activity in isolated muscle has been validated (25).

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

RESULTS

In vitro effects of GSK-3 inhibitor CT-98014 or lithium on GS activity. We initially determined the effectiveness of CT-98014 in inhibiting GSK-3 in isolated skeletal muscle preparations by use of an increase in GS₆ activity as the biomarker. As shown in Fig. 1, 500 nM CT-98014 caused increases of 51 and 48% in GS₆ activity in the epitrochlearis and soleus muscles, re-
spectively, of the lean Zucker rat. These increases due to CT-98014 were comparable to those elicited by 2 mU/ml insulin in these muscles (47 and 78%). The effects of CT-98014 and insulin on activation of GS_I in these muscles of the lean Zucker rats were completely additive. The total activity of GS was not altered in these muscles, due to the interventions (data not shown).

Insulin elicited small, but significant, increases in the GS_I activity of epitrochlearis (24%) and soleus (23%) muscles of the ZDF rat, reflecting the insulin-resistant state of these muscles. In contrast, CT-98014 elicited a completely normal activation of GS_I in these insulin-resistant muscles (49 and 50%). Moreover, when CT-98014 and insulin were present in combination, the activation of GS was substantially more than could be accounted for by summing the individual effects of the stimuli in the epitrochlearis (0.776 nmol·mg⁻¹·min⁻¹ vs. theoretical additive value of 0.662) and the soleus (1.31 vs. 1.11). The total activity of GS was again not altered, due to the interventions (data not shown). The total GS activities in the epitrochlearis (1.69 ± 0.04 vs. 1.26 ± 0.05 nmol·mg⁻¹·min⁻¹) and soleus (2.41 ± 0.06 vs. 1.63 ± 0.05 nmol·mg⁻¹·min⁻¹) were significantly greater in ZDF rats compared with lean Zucker rats, consistent with previous findings in the obese Zucker rat (30).

The effects of 10 mM lithium on the GS_I activity in muscle from lean Zucker and ZDF rats are shown in Fig. 2. Lithium significantly activated GS_I in epitrochlearis and soleus muscles of the ZDF rat, reflecting the basal glucose transport activity in epitrochlearis and soleus muscles of the lean Zucker rat (Fig. 3). In contrast, 10 mM lithium elicited significant increases in both basal glucose transport and insulin-stimulated glucose transport activity in skeletal muscle from the ZDF rat.

**In vitro effects of GSK-3 inhibitor CT-98014 or lithium on glucose transport activity.** CT-98014 (500 nM) had no effect on basal glucose transport or on submaximally or maximally insulin-stimulated glucose transport activity in epitrochlearis and soleus muscle from the lean Zucker rat. In contrast, 10 mM lithium elicited significant increases in both basal glucose transport and insulin-stimulated glucose transport activity in skeletal muscle from the lean Zucker rats.

CT-98014 at 500 nM also did not cause an activation of basal glucose transport in skeletal muscle of the ZDF rats, whereas 10 mM lithium alone stimulated basal glucose transport in both epitrochlearis and soleus muscles of these insulin-resistant animals (Fig. 4). Importantly, 500 nM CT-98014 caused significant enhancements of glucose transport activity in the presence of submaximally effective (37 and 43%) and maximally effective (24 and 27%) concentrations of insulin in epitrochlearis and soleus (27) muscles of ZDF rats. These significant effects of CT-98014 on insulin-stimulated glucose transport activity in muscle of the ZDF rat were observed at concentrations as low as 100 nM, with no greater maximal effects with concentrations as...
high as 1 μM (data not shown). Lithium at 10 mM also significantly enhanced glucose transport activity in ZDF muscle incubated with submaximally and maximally effective insulin concentrations, although most of this effect could be attributed to the activation of basal glucose transport (Fig. 4).

Fig. 2. Interactions of insulin and lithium on glycogen synthase (GS) activity in skeletal muscle of lean Zucker and ZDF rats. Epitrochlearis and soleus muscles were incubated with 2 mU/ml insulin and 10 nM lithium for 60 min. Values are means ± SE for 5 muscles per group. *P < 0.05 vs. control group; #P < 0.05 vs. all other groups.

In vitro effects of GSK-3 inhibitor CT-98014 on cell surface GLUT4. To gain some insight into the molecular mechanism for the effects of CT-98014 on glucose transport, we assessed the level of exofacial GLUT4 protein, using the impermeable photolabel 2-N-[4-(1-azi-2,2,2-trifluoroethyl)benzonyl]-1,3-bis(D-mannos-4-yloxy)-2-

Fig. 3. Effects of GSK-3 inhibitor CT-98014 or lithium on glucose transport activity in skeletal muscle of lean Zucker rats. Epitrochlearis and soleus muscles were incubated with insulin and 500 nM CT-98014 or 10 mM LiCl for 60 min. Values are means ± SE for 5 muscles per group. Soleus data with CT-98014 are adapted from Ring et al. (27). *P < 0.05 vs. minus lithium group.
propylamine (ATB-[2-3H]BMPA) (ATB-[3H]BMPA; Fig. 5). In the absence of insulin, CT-98014 did not alter cell-surface GLUT4 in either epitrochlearis or soleus muscles of the ZDF rat. Insulin caused significant increases in cell-surface GLUT4 in these muscles. Moreover, the insulin-stimulated increases in cell-surface GLUT4 were significantly enhanced by CT-98014 in both the epitrochlearis (29%) and the soleus (21%) muscles of the ZDF rat.

Effects of oral administration of GSK-3 inhibitor CT-98023 on glucose tolerance. The acute oral administration of the GSK-3 inhibitor CT-98023 (30 mg/kg body wt) led to substantial increases of the compound in plasma and skeletal muscle of the ZDF rats 30 min after completion of the treatments (Fig. 6), indicating that the metabolic actions of the compound in vivo should not be limited by bioavailability. Thirty minutes after the completion of these treatments, an OGTT was performed, and the responses of plasma glucose and insulin were assessed (Fig. 7). In vehicle-treated ZDF rats, the glucose feeding led to a large increase in plasma glucose at 30 min, and this glucose response was substantially reduced in the GSK-3 inhibitor-treated animals. The total glucose area under the curve (AUC) was 26% less in the GSK-3 inhibitor-treated ZDF rats than in the vehicle-treated control group (Fig. 8). Moreover, the insulin response during the OGTT was also dramatically reduced in the GSK-3 inhibitor-treated group, and the total insulin AUC was 34% less in the GSK-3 inhibitor-treated ZDF rats than...
in the control group. These decreases in the glucose and insulin responses of the OGTT following the GSK-3 inhibitor treatment were even more pronounced when the incremental AUCs for glucose (13,620 ± 1,216 vs. 6,600 ± 644 mg·dl⁻¹·min) and insulin (4,290 ± 540 vs. 1,020 ± 205 μU·ml⁻¹·min) were calculated. The dose of GSK-3 inhibitor used in this in vivo study (30 mg/kg) is maximally effective in enhancing oral glucose tolerance, as a higher dose (48 mg/kg) does not have any further effect (27).

The glucose-insulin index is defined as the product of the glucose and insulin AUCs, and a reduction in this value represents indirect evidence of improved in vivo peripheral insulin action (6). After acute treatment with CT-98023, the glucose-insulin index was reduced by 51% compared with the vehicle-treated group, consistent with an increase in whole body insulin sensitivity. This decrease in the glucose-insulin index due to GSK-3 inhibition was also observed when the incremental AUCs were used (58 ± 12 vs. 7 ± 1 units × 10⁶).

**Effects of oral administration of GSK-3 inhibitor CT-98023 on muscle glucose transport.** To identify a potential cellular locus for the improved peripheral insulin sensitivity, insulin-stimulated glucose transport activity was determined in epitrochlearis and soleus muscles of the ZDF rats at various time points after the acute administration of CT-98023 (Fig. 9). Thirty minutes after the completion of the oral GSK-3 inhibitor treatment, the maximal insulin-mediated increase in glucose transport activity was enhanced in both the epitrochlearis (56%) and soleus (43%) compared with the corresponding control groups. At this same time point, glucose transport activity due to a submaximally effective concentration of insulin (150 μU/ml) was similarly enhanced in these muscles from the CT-98023-treated ZDF rats (data not shown). Two hours after the oral administration of CT-98023, the enhanced action of the maximally effective concentration of insulin was still detectable in the soleus muscle (26%) but not in the epitrochlearis muscle, consistent with the diminished glucose-lowering effect of the treatment at this time point (Fig. 7). The enhanced insulin action on glucose transport activity due to the GSK-3 inhibitor treatment was no longer detectable in the soleus after 4 h.

**DISCUSSION**

We have demonstrated in the present investigation that the selective inhibition of GSK-3 in insulin-resistant skeletal muscle of the ZDF rat was associated with a potentiation of the ability of insulin to stimulate both GS activity (Fig. 1) and glucose transport activity (Fig. 3). These effects of the GSK-3 inhibitor CT-98014 were elicited at concentrations in the nanomolar range, with maximal effects at 500 nM. Interestingly, although the GSK-3 inhibitor CT-98014 clearly activated GS activity in skeletal muscle from the insulin-sensitive lean Zucker rat (Fig. 1), it had absolutely no effect on either basal or insulin-stimulated glucose transport activity in muscle from these lean animals. These results support the idea that an elevation in GSK-3 activity, which exists in muscle of the ZDF rat but not the lean rat (2), is necessary for a cell to respond to GSK-3 inhibition for enhancement of insulin-stimulated glucose transport activity.
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 Nikoulina et al. (21), who showed in cultured human myocytes that these same GSK-3 inhibitors, when administered acutely (30 min), can upregulate insulin-stimulated GS activity and, when administered chronically (4 days), can enhance insulin-mediated glucose transport activity. Interestingly, these investigators also demonstrated that chronic treatment of cultured human muscle cells with GSK-3 inhibitor caused a downregulation of GSK-3 protein expression and activity and a large upregulation of IRS-1 protein level. Whether the chronic administration of GSK-3 inhibitor causes similar effects in insulin-resistant muscle from rodent models or humans remains to be determined.

At least part of the beneficial effect of the GSK-3 inhibitor CT-98014 on insulin-stimulated glucose transport activity in skeletal muscle of the ZDF rat was due to an enhancement of GLUT4 protein at the cell surface (Fig. 5). This is in contrast to the finding that the nonspecific GSK-3 inhibitor lithium did not enhance insulin-stimulated GLUT4 translocation in 3T3-L1 adipocytes overexpressing GSK-3β (33). The underlying molecular mechanism for the increased GLUT4 translocation in response to the GSK-3 inhibition in insulin-resistant skeletal muscle was not addressed in the present study. However, it is known that intact activation of PI 3-kinase by tyrosine-phosphorylation...
lated IRS-1 is necessary for this insulin-stimulated GLUT4 translocation to take place (reviewed in Ref. 38). Because GSK-3 is known to inhibit IRS-1 action by serine/threonine phosphorylation (10), one possibility for the beneficial effect of the GSK-3 inhibitor is through a disinhibition of IRS-1 functionality, with a subsequent enhancement of PI 3-kinase activity and increased GLUT4 translocation. It is clear that this would be a fruitful area for future investigations.

We also compared the metabolic effects of lithium, which has been used previously as an inhibitor of GSK-3, with those of the more selective GSK-3 inhibitor CT-98014 (Figs. 1–4). Both lithium (at 10 mM) and CT-98014 (at 500 nM) elicited enhancement of GS activity in muscle from lean Zucker and ZDF rats and displayed an additive interaction with insulin for stimulation of GS activity in muscle from lean animals. This additivity between lithium and insulin for activation of GS in insulin-sensitive cells is consistent with previous studies (3, 6, 23, 33). Moreover, lithium and CT-98014 both displayed a synergistic interaction with insulin for stimulation of GS activity and glucose transport activity in muscle from ZDF rats. However, there were some striking differences between lithium and CT-98014 concerning stimulation of glucose transport activity. Although CT-98014 had no effects on glucose transport activity in the absence of insulin in both the lean Zucker and the ZDF groups or on insulin-stimulated glucose transport activity in the lean group, lithium caused an increase in basal glucose transport in both groups and in insulin-stimulated glucose transport activity in the lean group. Whereas lithium can certainly inhibit GSK-3, this ion obviously has additional effects that must account for its actions on basal and insulin-stimulated glucose transport in insulin-sensitive skeletal muscle and on basal glucose transport activity in insulin-resistant skeletal muscle. Therefore, one must exercise caution in using lithium as an inhibitor of GSK-3 in skeletal muscle studies, as other effects of this ion apart from those on GSK-3 may confound interpretation of the results.

There were some important differences in the glucose transport responses of the epitrochlearis, which consists primarily of type I fibers (20, 29), and the soleus, which consists mainly of type I fibers (1), to the GSK-3 inhibitor CT-98014 (Fig. 4). The absolute increase in insulin-stimulated glucose transport activity due to GSK-3 inhibition was 34–37 pmol·mg⁻¹·20 min⁻¹ in the epitrochlearis, whereas in the soleus this increase was substantially greater (122–126 pmol·mg⁻¹·20 min⁻¹). This greater response to GSK-3 inhibition in the soleus may reflect the fact that type I fibers express a much higher level of insulin-signaling factors (31) and GLUT4 protein (13, 17) than type IIb fibers and therefore may have a higher capacity for insulin signaling once inhibition of one of these factors [e.g., GSK-3 inhibition of IRS-1 (10)] is removed.

The oral administration of GSK-3 inhibitors to the insulin-resistant ZDF rat also elicited improvements in glucose metabolism. The disposal of an oral glucose load by the ZDF rats acutely treated with the GSK-3 inhibitor CT-98023 was markedly enhanced compared with the vehicle-treated animals (Figs. 7 and 8). One potential cellular locus for this increased glucose disposal was skeletal muscle, as insulin-stimulated glucose transport activity in both the epitrochlearis and the soleus was substantially increased at the 30-min time point, when the glucose response during the OGTT was maximally reduced (Fig. 7). Indeed, at the 120-min time point following the final administration of the GSK-3 inhibitor, the enhancement of insulin-stimulated glucose transport activity was no longer observed in the epitrochlearis and was substantially diminished in the soleus (Fig. 9), whereas at this same time point the glucose levels during the OGTT were not different between the GSK-3 inhibitor-treated and the vehicle-treated control animals (Fig. 7). This apparent reversibility of the GSK-3 inhibition is consistent with the observation that removal of the compound from tumor cells causes β-catenin levels to decrease (Harrison SD, unpublished data).

One additional potential site of action of GSK-3 inhibitors that could contribute to an improvement in whole body glucose homeostasis is the liver (4, 19). Cline et al. (4) have recently reported that treatment of ZDF rats with the selective GSK-3 inhibitor CT-98023 significantly enhances GS activity and glycogen synthesis in the liver. Moreover, Lochhead et al. (19) have demonstrated that the selective reduction of GSK-3 activity in rat hepatoma cells by treatment with lithium or cell-permeable, small-molecular-weight compounds (SB-216763 and SB-415286) (5) is associated with a reduction in the expression of the gluconeogenic enzymes phosphoenolpyruvate carboxykinase and glucose-6-phosphatase. Therefore, an enhancement of hepatic glycogen synthesis and a reduction in hepatic glucose output likely contribute to the glucose-lowering effect of GSK-3 inhibitors in rodent models of type 2 diabetes (27).

In summary, we have demonstrated that direct in vitro exposure of insulin-resistant type I and type IIb skeletal muscle from the ZDF rat to a novel, selective organic inhibitor of GSK-3 can potentiate the action of insulin to stimulate GS activity and glucose transport. Although lithium also potentiated insulin-stimulated GS activity and glucose transport in insulin-resistant muscle, it additionally elicited effects on basal glucose transport and on insulin-stimulated glucose transport in muscle from lean Zucker rats that were not affected by the selective GSK-3 inhibitor. The effect of the GSK-3 inhibitor on insulin-stimulated glucose transport in skeletal muscle from the ZDF rat was due, at least in part, to an enhanced cell surface GLUT4 protein level. Moreover, the acute oral administration of a selective GSK-3 inhibitor caused a significant improvement in whole body glucose disposal and insulin sensitivity that was associated with enhanced skeletal muscle glucose transport activity. This novel approach using selective organic GSK-3 inhibitors appears useful as a pharmacological treatment against insulin resistance of skeletal muscle glucose disposal.
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