Effects of a Novel Glycogen Synthase Kinase-3 Inhibitor on Insulin-Stimulated Glucose Metabolism in Zucker Diabetic Fatty (*fa/fa*) Rats

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Defects in liver and muscle glycogen synthesis are major factors contributing to postprandrial hyperglycemia in patients with type 2 diabetes. Therefore, activation of glycogen synthase through inhibition of glycogen synthase kinase (GSK)-3 represents a potential new therapeutic target. To examine this possibility, we performed oral glucose tolerance tests (OGTTs) and euglycemic-insulinemic clamp studies in Zucker diabetic fatty (*fa/fa*) rats before and after treatment with novel GSK-3 inhibitors. GSK-3 inhibition caused a 41 ± 2% ($P < 0.001$) and 26 ± 4% ($P < 0.05$) reduction in the area under the glucose and insulin concentration curves, respectively, during the OGTT. This improvement in glucose disposal could mostly be attributed to an approximate twofold increase in liver glycogen synthesis. In contrast, there was no significant increase in muscle glycogen synthesis despite an approximate threefold activation of muscle glycogen synthase activity. GSK-3 inhibitor treatment increased liver glycogen synthesis about threefold independent of insulin concentration during the clamp studies. In contrast, muscle glucose uptake and muscle glycogen synthesis were independent of drug treatment. GSK-3 inhibitor treatment lowered fasting hyperglycemia in diabetic rats by 6.0 ± 1.3 mmol/l but had no significant effect on glucose disposal during the clamp. In conclusion, GSK-3 inhibition significantly improved oral glucose disposal, mostly by increasing liver glycogen synthesis. These studies suggest that GSK-3 inhibition may represent an important new therapeutic target for treatment of patients with type 2 diabetes. *Diabetes* 51:2903–2910, 2002

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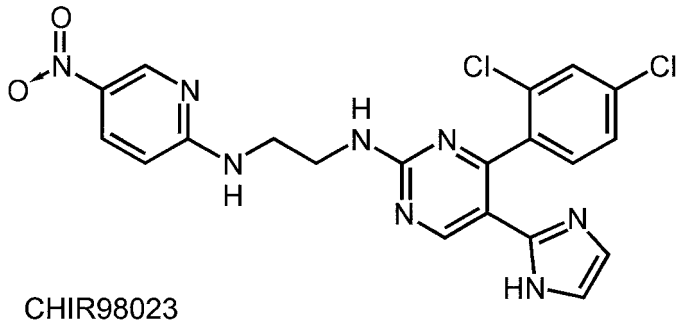
EC_{50}, half-maximal effective concentration; EGP, endogenous glucose production; G6P, glucose-6-phosphate; GSK, glycogen synthase kinase; [^{3}H]2DG, 2,6-[^{3}H]-2-deoxy-D-glucose; IC_{50}, half-maximal inhibitory concentration; OGTT, oral glucose tolerance test.

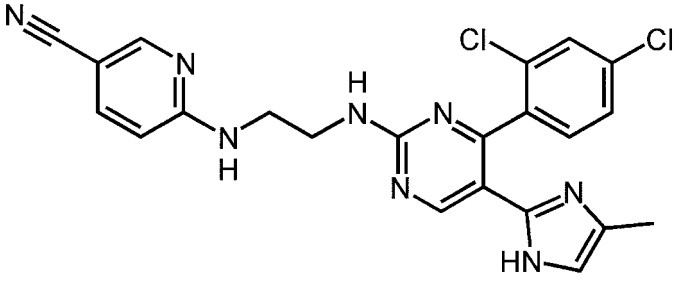
Defects in insulin-stimulated liver and muscle glycogen synthesis are major factors contributing to postpandrial hyperglycemia in patients with type 2 diabetes (1–11). Increasing the activity of glycogen synthase may therefore provide a means to promote glycogen synthesis, and hence glucose disposal, in type 2 diabetic patients. Glycogen synthase activity is reduced by coordinated phosphorylation at any of several potential serine residues by glycogen synthase kinase (GSK)-3, with glucose-6-phosphate (G6P) providing an additional level of allosteric control independent of the phosphorylation state (12–14). Therefore, inhibition of GSK-3 activity leads to activation of glycogen synthase activity and may enhance glucose uptake.

In addition to insulin, Li^+, at pharmacological concentrations, has been shown to be an effective inhibitor of GSK-3β and, in vitro, stimulated glucose flux into glycogen, either alone or combined with insulin. Although Li^+ also enhanced glucose uptake in hepatocytes, adipocytes, and, to a lesser extent, in muscle (15–19), inactivation of GSK-3β by Li^+ had no effect on GLUT4 translocation (16) and by itself had minimal effect on rates of glucose uptake in muscle (17,18). The results from recent studies have led to the hypothesis that the locus of control for insulin-stimulated glucose disposal in muscle is primarily at glucose transport, whereas in liver, glucose transport is not rate-limiting (8,10). The development of highly specific GSK-3 inhibitors (CHIR98023 and CHIR99021) provided us with another tool to test the hypothesis that insulin resistance in muscle is due to impaired glucose uptake, as opposed to defects in the regulation and activity of glycogen synthase.

Recent in vitro studies of cultured human skeletal muscle chronically treated for 4 days with the GSK-3 inhibitors CHIR98014 and CHIR98023 showed significant increases in both basal and insulin-stimulated glucose uptake (20). Furthermore, ex vivo studies of glucose uptake by soleus muscle of diabetic rats support the hypothesis that GSK-3 inhibition may stimulate muscle glycogen synthesis (21). In addition, inhibitors of GSK-3 have been shown to suppress the expression of the gluconeogenic enzymes PEPCK and glucose-6-phosphatase in vitro (22). This may have the added benefit of reducing hepatic glucose production. We have therefore evaluated the effects of novel GSK-3 inhibitors on glucose



CHIR98023



CHIR99021

FIG. 1. Molecular structures of the GSK-3β inhibitors CHIR98023 and CHIR99021.

disposal and endogenous glucose production (EGP), after an oral glucose tolerance test (OGTT) and during hyperinsulinemic-euglycemic clamps, in both awake insulin-resistant prediabetic and overtly diabetic Zucker diabetic fatty (ZDF) (*fa/fa*) rats.

RESEARCH DESIGN AND METHODS

Animals. For the OGTTs and the acute (4-h) treatment studies, male ZDF (*fa/fa*) rats (Genetic Models, Indianapolis, IN) weighing between 260 and 320 g were studied at 9–10 weeks of age before the onset of overt diabetes. For the chronic (20-h) treatment studies, male ZDF (*fa/fa*) rats weighing between 370 and 430 g were studied at 13 weeks of age, after the onset of diabetes (as determined by fasting blood glucose between 8.3 and 12.5 mmol/l). They were maintained on Purina 5008 rat chow (Ralston-Purina, St. Louis, MO) and housed in an environmentally controlled room with a 12-h light/dark cycle. One week before the study, an internal jugular catheter and a carotid artery catheter were implanted, as previously described (23). The catheters were externalized through a skin incision at the back of the head. All rats were fasted for 16–18 h before the study. All experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, and all protocols were approved by the Yale Animal Care and Use Committee.

GSK-3 inhibitors. We used CHIR98023 and CHIR99021 during the course of these experiments. Both inhibitors are low molecular weight compounds with closely related structures (Fig. 1). CHIR99021 potency (half-maximal inhibitory concentration [IC_{50}] 20 nmol/l, half-maximal effective concentration [EC_{50}] 766 nmol/l) and selectivity (>10,000×) are approximately twice that of CHIR98023 (IC_{50} 10 nmol/l, EC_{50} 375 nmol/l), as determined by cell-free and cell-based assays (21). The pharmokinetic parameters were determined in ZDF rats by intravenous dosing with 30 mg/kg CHIR98023 or CHIR99021. Administration of the CHIR compounds, by oral or intravenous routes, results in good peripheral (not central nervous system) tissue distribution, including major glucose-regulating tissues such as liver, muscle, and even adipose. The volume of distribution was 3 l/kg, with a half-life of 90 min, a clearance rate of 75 ml · min^{-1} · kg^{-1}, and a tissue-to-plasma concentration ratio of ~0.25 in muscle and ~7 in liver.

Validation of direct GSK-3 inhibition in vivo is technically challenging with reversible/competitive inhibitors such as the specific CHIR molecules. Treatment with the inhibitors followed by homogenate preparation and enzyme immunoprecipitation results in washout of distributed compound and resumption of enzyme activity on analysis. One is confined to measuring downstream indicators of GSK-3 inhibition (glycogen synthase activation and phosphorylation changes, B-catenin elevation, and glycogen formation). In this context, we have immunoprecipitated GSK-3 from naïve ZDF rat muscle and liver with polyclonal rabbit antisera and tested GSK-3 inhibition ex vivo and found K_i = 5 nmol/l (CHIR98023) and K_i = 10 nmol/l (CHIR99021). For other indicators of GSK-3 activity, we have measured B-catenin changes in CHIR99021-infused ZDF rats and have observed a ~50% increase in certain tissues, such as gastrointestinal tract, but have found that end point to be less sensitive than glycogen content.

OGTT. At −270 and −30 min, the rats were gavaged with either the GSK-3 inhibitor CHIR98023 (30 mg/kg) (Chiron, Emeryville, CA) dissolved in saline with 1% captisol as the vehicle (n = 13) or 1% captisol (bolus 10%) dissolved in saline alone (n = 13). At 0 min, the rats were given 4 ml/kg of 50% dextrose (~30% enriched with [1-^{13}C]glucose by gavage). The fraction of liver glycogen synthesized via the direct pathway was assessed by administering acetaminophen (30 mg/kg) dissolved in the glucose solution (24). Plasma samples were taken before and every 15 min after the OGTT for determination of blood glucose concentration and ^{13}C enrichment of plasma glucose and acetaminophen-glucuronide. At 120 min, the animals were anesthetized by an intravenous administration of pentobarbital (50 mg/kg rat) and the quadriceps and gastrocnemius muscles were freeze-clamped in situ and then excised. Livers were then quickly removed and freeze-clamped with Wollenberger clamps precooled in liquid nitrogen.

Hyperinsulinemic-euglycemic clamps (acute effects of GSK-3 inhibition). After an overnight fast, and 2 h before the start of the clamp, the animals received either a primed (6.3 mg/kg) continuous (0.075 mg · kg^{-1} · min^{-1}) infusion of CHIR99021 (n = 6) in a saline solution with 1% captisol or a primed continuous infusion of the saline 1% captisol solution (n = 8). To assess EGP during the basal period and throughout the clamp procedure, a tracer infusion (0.36 μmol · kg^{-1} · min^{-1}) of [U-$^{13}C_6$]glucose (99% U-$^{13}C_6$) was started simultaneously with the drug/vehicle infusion. After a 2-h tracer and drug/vehicle infusion, the animals underwent a 3-h hyperinsulinemic-euglycemic clamp at either a submaximal insulin dose of 2 mU · kg^{-1} · min^{-1} or a maximal insulin dose of 20 mU · kg^{-1} · min^{-1}, as previously described (23). A variable infusion of [1-^{13}C]glucose (20% ^{13}C) was infused to maintain plasma glucose at ~5 mmol/l and to calculate rates of glycogen synthesis. Venous plasma samples (0.2 ml) were taken every 20 min to determine glucose concentration, [^{13}C]glucose enrichment, insulin concentration, and drug concentration.

Forty-five minutes before the end of the clamp, relative rates of muscle glucose uptake were determined from the uptake of 2,6-[^{3}H]-2-deoxy-D-glucose, ([^{3}H]2DG) (NEN Life Science Products, Boston, MA) (25). The animals received a bolus intravenous injection of [^{3}H]2DG (50 μCi) chased with saline. Blood samples were taken to define the specific activity exponential decay curve, with frequent samples taken during the first 10 min and continuing until termination of the experiment. The animals were then anesthetized by an intravenous injection of sodium pentobarbital and the quadriceps and liver tissues were freeze-clamped in situ and stored at −80°C for further analysis.

Hyperinsulinemic-euglycemic clamps (chronic effects of GSK-3 inhibition). On day 0, an infusion of CHIR99021 (n = 7; prime 10 mg/kg, constant infusion 0.026 mg/min) or vehicle (n = 7) was begun at 11:00 A.M. and continued until the end of the hyperinsulinemic-euglycemic clamp. Food was withdrawn immediately before the start of the infusion. At 9:00 A.M. of day 1, the animals underwent a 90-min hyperinsulinemic-euglycemic clamp at an insulin dose of 8 mU · kg^{-1} · min^{-1}, as described above. Blood samples were taken immediately before and every 10 min throughout the clamp. In a second group of animals (CHIR99021 treated, n = 6; controls, n = 6), a tracer infusion of [U-$^{13}C_6$]glucose was begun 2 h before the start of the insulin infusion. At the end of the clamp period, the animals were killed with pentobarbital (50 mg/kg rat) and the liver, quadriceps muscle, and gastrocnemius muscle were freeze-clamped in situ and stored at −80°C for further analysis.

Analyses. Tissue glycogen was isolated and digested with amyloglucosidase (Sigma, St. Louis, MO). Plasma glucose and digested glycogen concentrations were determined by the glucose oxidase reaction (Glucose Analyzer II; Beckman Instruments, Fullerton, CA). Immunoreactive insulin was assayed using a double-antibody immunoassay kit and rat insulin standards (Linco Research, St. Louis, MO). Plasma concentrations of the GSK-3 inhibitors CHIR98023 and CHIR99021 were determined by high-performance liquid chromatography.

Isotopic analysis. The percentage of liver glycogen synthesized by the direct pathway (%Gly_{DIR}; glucose→G6P→glucose-1-phosphate→UDP-glucose→glycogen) was calculated from the ratio of ^{13}C enrichment in C1 of plasma acetaminophen and plasma glucose (24). The enrichments were determined by gas chromatography–mass spectrometry using an HP5890-MSD5971 (Hewlett-Packard, Palo Alto, CA) analysis of the penta-acetate derivative of

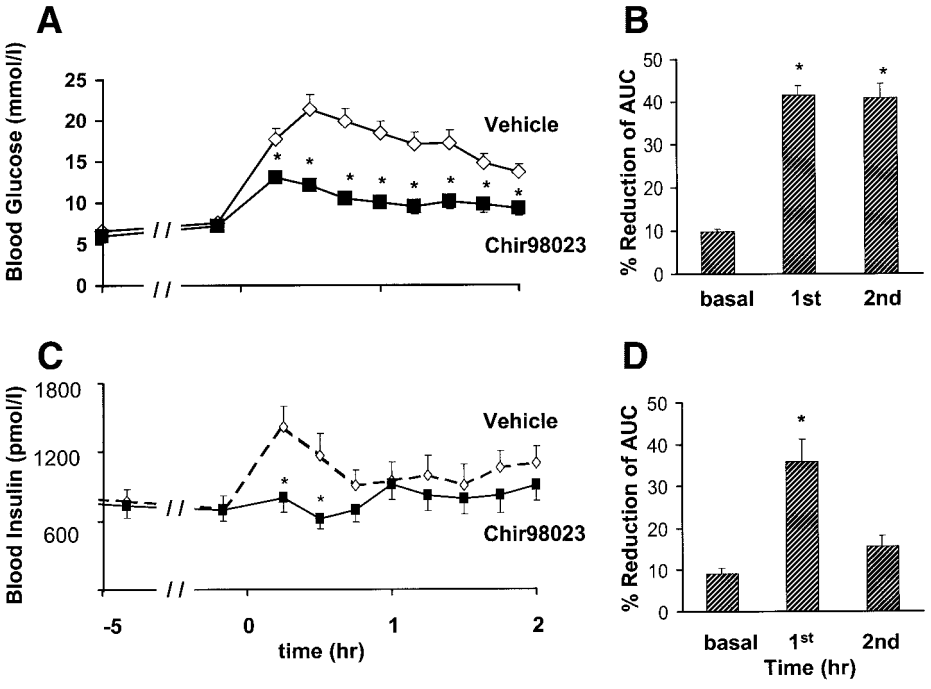


FIG. 2. Mean blood glucose concentration (*A*) and blood insulin concentrations (*C*) in ZDF (*fa/fa*) rats treated with vehicle (◇) or CHIR98023 (■) after an OGTT, and the percent reduction in the area under the curve (AUC) of blood glucose concentration (*B*) and blood insulin concentration (*D*) of the CHIR98023-treated rats compared with vehicle-treated rats. *$P < 0.05$.

glucose and the glucose moieties of glycogen (m/z [charge/mass ratio] 169–171 and 200–202 in electron impact mode). The difference between these two fragment ions gives the enrichment in C1. Gas chromatography–mass spectrometry analysis of the n-butyl ester, tri-ester derivative of acetaminophen-glucuronide (m/z 359–370 in chemical ionization mode) was used to obtain the total enrichment, and ^{13}C-NMR was used to determine the relative distribution of ^{13}C in the carbon skeleton.

Glycogen synthase activity. Glycogen synthase activity was measured in tissue homogenates as previously described (26,27). The percentage of maximal activity of glycogen synthase was defined as the rate of incorporation of [U-^{14}C]uridine diphosphoglucose into glycogen at physiological concentration of the enzyme's activator, G6P, divided by maximal glycogen synthase activity measured at saturating G6P concentration (7.2 mmol/l).

Rates of glycogen synthesis. Glycogen synthesis rates were calculated from the difference of the glycogen concentration measured at the end of the OGTT or clamp and from fasting glycogen concentrations measured in a separate group of rats. Alternatively, the net mass of glycogen synthesized during the OGTT and clamp studies was calculated from the product of the final glycogen concentration ($[Gly]_{FINAL}$), the percentage of the glycogen synthesized during the studies ($\%Gly_{SYN}$), and the percentage of glycogen synthesized directly from plasma glucose ($\%Gly_{DIR}$) as follows: mass of glycogen synthesized = $[Gly]_{FINAL} \times (\%Gly_{SYN}/100)/(\%Gly_{DIR}/100)$. The percentage of glycogen synthesized during the studies was calculated as: $\%Gly_{SYN} = 100 \times ([1\text{-}^{13}C]glycogen/[1\text{-}^{13}C]glucose)$. The percentage of glycogen synthesized directly from plasma glucose was calculated as: $\%Gly_{DIR} = 100 \times ([1\text{-}^{13}C]acetaminophen\text{-}glucuronide/[1\text{-}^{13}C]glucose)$.

The direct pathway was calculated, allowing for ~60 min lag time from conjugation to appearance in plasma of acetaminophen-glucuronide. The change in tissue glycogen content was extrapolated to total glycogen content using a mean liver weight of 16 ± 2 g and assuming that skeletal muscle represented 40% of total body weight (28,29).

Rates of muscle glucose uptake. Muscle glucose uptake rates were calculated from the disappearance rate of plasma [^{3}H]2DG into phosphorylated [^{3}H]2DG in muscle, as previously described. (25)

Statistical analyses. All data are reported as means ± SE. Unpaired two-tailed Student's *t* tests were used for comparisons between groups. Paired two-tailed Student's *t* tests were used to compare the results within an individual rat. Differences were considered statistically significant at $P < 0.05$.

RESULTS

OGTTs. Oral administration of CHIR98023 (30 mg/kg) to insulin-resistant ZDF (*fa/fa*) rats raised plasma concentrations of the inhibitor to 2–6 μmol/l and significantly ($P < 0.01$) reduced the plasma glucose concentrations during both the first and second hour after the OGTT. The integrated area under the plasma glucose curve was reduced by 41 ± 3% compared with the control animals (Fig. 2). The reduction of plasma glucose concentration in the CHIR98023-treated animals was accompanied by a similar reduction in plasma insulin concentrations during the first hour ($P < 0.01$), with the trend continuing into the second hour (Fig. 2), reflecting improved insulin sensitivity.

The effect of CHIR98023 on rates of glycogen synthesis was assessed with ^{13}C-labeled glucose to monitor newly synthesized muscle and liver glycogen and with acetaminophen to determine the pathways of liver glycogen synthesis. We measured a small but nonsignificant decrease in the percent direct flux of plasma glucose into the liver of CHIR98023-treated rats (45 ± 3%) compared with the vehicle-treated rats (56 ± 3%, $P = 0.077$). However, CHIR98023 treatment, which was associated with a threefold increase in liver glycogen synthase activity (Fig. 3), caused a twofold increase in the total amount of new glycogen deposited in the liver (Fig. 4). Liver glycogen concentration was increased by 43 ± 6 mmol/kg liver in the control rats compared with 85 ± 11 mmol/kg liver in the CHIR98023-treated rats ($P = 0.01$) after 2 h. If we extrapolate this to the total amount of liver synthesized using a mean liver weight of 16 g, then we calculate that the control rats stored 21 ± 3% of the glucose load as liver glycogen, which is quite similar to previously reported values in the rat and dog (30–34). In the CHIR98023-treated rats, 42 ± 5% of the glucose load was stored as liver glycogen.

In contrast to the stimulation of glycogen synthesis in liver by inhibition of GSK-3, we did not see any significant difference between the groups in the amount of muscle glycogen synthesized after the OGTT (Fig. 4) despite a threefold increase in muscle glycogen synthase activity (Fig. 3). Muscle glycogen concentration was increased by 5.0 ± 0.6 mmol/kg muscle in the control rats compared with an increase of 6.1 ± 0.8 mmol/kg muscle in the CHIR98023-treated rats. When extrapolated to the whole

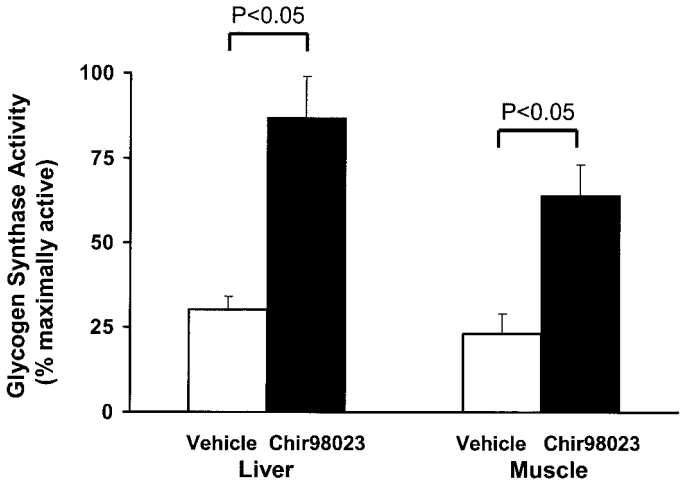


FIG. 3. Percent of maximally active form of glycogen synthase in liver and muscle of ZDF (*fa/fa*) rats treated with vehicle (□) or CHIR98023 (■) after an OGTT. *$P < 0.05$.

body, assuming that muscle mass is 40% of total body weight (28,29), we calculated that the glucose load stored as muscle glycogen (control 10.9 ± 1.2%, CHIR98023 treated 13.2 ± 1.7%) was one-half or less that stored as liver glycogen.

Although we have not accounted for all possible tissue fates (e.g., gut, brain, and adipose) of the glucose load, it is likely that the increase from ~20 to ~40% of the fraction stored as liver glycogen is primarily responsible for the enhanced disposal of the oral glucose load in the treated animals.

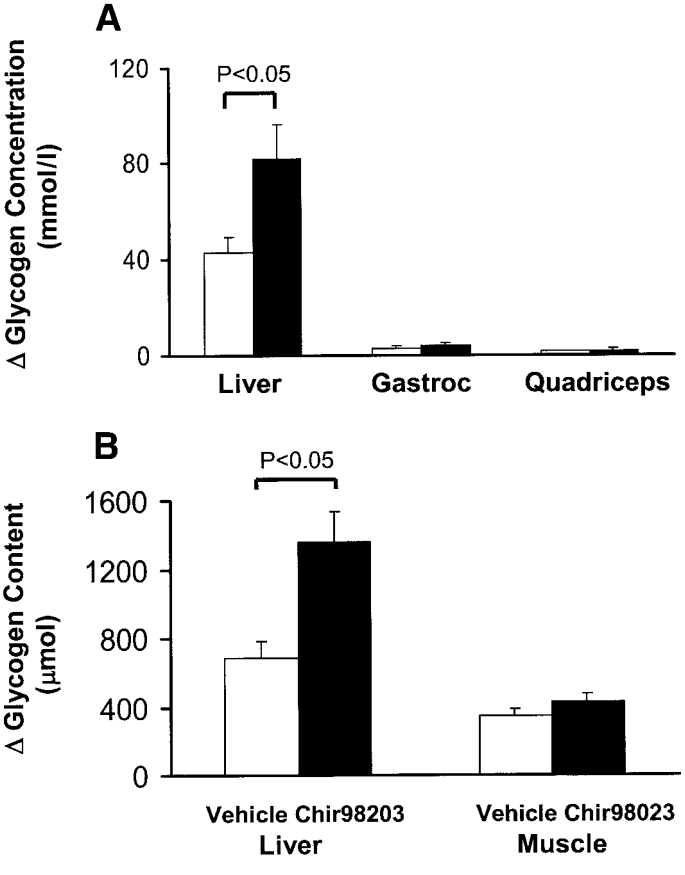


FIG. 4. *A*: Change in glycogen concentration in liver, gastrocnemius, and quadriceps muscle in ZDF (*fa/fa*) rats treated with vehicle (□) or CHIR98023 (■) after an OGTT. *B*: Change in total glycogen content was calculated using a mean liver weight of 16 g and assuming that skeletal muscle represented 40% of total body weight.

Hyperinsulinemic-euglycemic clamps

Acute effects of GSK-3 inhibition in prediabetic rats. Infusion of CHIR99021 at 0.075 mg · kg^{-1} · min^{-1} resulted in a sustained CHIR99021 plasma concentration of ~5 μmol/l, a concentration that is about sevenfold greater than its EC_{50} in cultured CHO-IR cells. Plasma glucose concentrations were matched, although CHIR99021-treated animals showed a nonsignificant trend toward lower plasma insulin concentrations at the higher insulin infusion rate (Table 1). Although CHIR99021 produces an acute OGTT response that is nearly identical to CHIR98023 (data not shown), it was used for the clamp studies because it had been optimized for intravenous infusion.

Whole-body glucose turnover. Short-term infusion of CHIR99021 had no significant effect on the glucose infusion rates necessary to maintain euglycemia at either the low (2 mU · kg^{-1} · min^{-1}), or high (20 mU · kg^{-1} · min^{-1}) insulin infusion rates (Table 1). From the [U-$^{13}C_6$]glucose infusion, we calculated similar glucose rates of appearance and EGP in the treated and untreated rats under basal conditions and during the insulin infusions (Table 1). At the low insulin infusion rate, there was a small but significant reduction of EGP in the CHIR99021-treated animals. At the higher insulin infusion rate, EGP was equally suppressed in both groups (controls 63 ± 11%, CHIR98023 treated 64 ± 15%).

Liver and muscle glycogen synthesis. At the euglycemic plasma levels in these studies, rates of liver glycogen synthesis were not affected by plasma insulin concentrations (Fig. 4). However, treatment with CHIR99021 increased liver glycogen synthesis approximately threefold at both the low and high doses of insulin (Fig. 5).

In contrast, the increase in plasma insulin concentrations led to a twofold higher rate of muscle glycogen (Fig. 5) synthesis and muscle glucose uptake (Fig. 6) in both the control and treated animals. CHIR99021 had no discernible effect on rates of either glucose uptake or glycogen synthesis in muscle.

Chronic effects of GSK-3 inhibition in diabetic rats. An overnight infusion of the GSK-3 inhibitor profoundly reduced ($P < 0.01$) fasting hyperglycemia in the diabetic ZDF (*fa/fa*) rats by lowering plasma glucose from 11.7 ± 1.3 mmol/l in the controls to 5.8 ± 1.2 mmol/l in the CHIR99021-treated rats (Fig. 7). During the clamps in this group of rats, CHIR99021 treatment had no significant effect on insulin responsiveness, as reflected by similar glucose infusion rates required to maintain euglycemia (Table 2).

In a separate group of rats receiving an overnight infusion of CHIR99021 and [U-$^{13}C_6$]glucose tracer, fasting blood glucose was reduced by 4.7 ± 1.3 mmol/l ($P < 0.05$) and basal EGP was reduced by 42% (Fig. 7). Although an overnight infusion of CHIR99021 lowered fasting plasma glucose by an amount comparable to that in the first group, diabetes was more advanced in these animals and fasting plasma glucose concentrations were significantly higher, precluding the possibility of performing euglycemic-insulinemic clamps on these animals using an 8-mU · kg^{-1} · min^{-1} insulin infusion rate.

TABLE 1
Effect of CHIR99021 treatment on plasma glucose and insulin concentrations, glucose infusion rate, glucose disposal, and EGP during euglycemic-hyperinsulinemic clamps in prediabetic ZDF (*fa/fa*) rats.

	Insulin Infusion rate			
	2 mU · kg^{-1} · min^{-1}		20 mU · kg^{-1} · min^{-1}	
	Control	CHIR99021 treated	Control	CHIR99021 treated
Glucose (mmol/l)				
Basal	5.7 ± 0.3	6.3 ± 0.3	6.8 ± 0.2	6.2 ± 0.2
Clamp	7.1 ± 0.3	6.8 ± 0.3	5.6 ± 0.2	5.8 ± 0.3
Insulin (pmol/l)				
Basal	535 ± 85	671 ± 78	571 ± 123	718 ± 135
Clamp	542 ± 59	660 ± 64	6513 ± 709*	6107 ± 386*
Glucose infusion rates (μmol · kg^{-1} · min^{-1})				
Basal	0	0	0	0
Clamp	8.3 ± 2.3	11.2 ± 3.1	96.8 ± 4.9	85.1 ± 5.0
Glucose disposal (μmol · kg^{-1} · min^{-1})				
Basal	83.4 ± 7.9	80.4 ± 9.8	72.1 ± 9.0	65.1 ± 12.1
Clamp	89.8 ± 9.1	78.4 ± 9.1	125.3 ± 12.0*	117.4 ± 12.0*
EGP (μmol · kg^{-1} · min^{-1})				
Basal	83.4 ± 7.9	80.4 ± 9.8	72.1 ± 9.0	65.1 ± 12.1
Clamp	81.6 ± 10.7	67.2 ± 9.1*	28.5 ± 9.7*	32.3 ± 16.7*

*

Data are means ± SE. *$P < 0.05$ compared to basal.

DISCUSSION

In addition to defects in glucose transport and phosphorylation (1,2,6–11), glycogen synthase activity in liver and muscle has been shown to be reduced and resistant to stimulation by insulin in patients with type 2 diabetes (3–5,33). Therefore, an attractive hypothesis for improving glucose disposal in these patients would be to increase the activity of glycogen synthase leading to increased rates of glycogen synthesis in these organs. To test this hypothesis and avoid the confounding effects of glucose toxicity on glucose disposal, we first characterized the effects of the novel GSK-3 inhibitors CHIR98023 and CHIR99021 in ZDF (*fa/fa*) rats before the onset of overt diabetes. The possibility of effectively treating fasting and postprandrial hyperglycemia with GSK-3 inhibitors was then tested in the overtly diabetic ZDF (*fa/fa*) rat.

The phosphorylation of glycogen synthase by the β-isoform of GSK-3 is a critical component in the regulation of glycogen synthase activity (13,14). Phosphorylation of glycogen synthase inhibits glycogen synthase activity. In insulin-responsive tissue, insulin exposure leads to a transient inhibition of GSK-3β via protein kinase B phosphorylation of a serine residue (Ser9) (12,13). The inhibition of GSK-3β activity leads to net dephosphorylation and hence activation of glycogen synthase. GSK-3β has also been shown to be involved in the regulation of several other metabolic and developmental processes (13,14). Other substrates include regulatory subunits of protein phosphatase-1 and cyclic AMP-dependent protein kinase, the transcription factors c-*jun* and c-*myc*, and those involved in the expression of the gluconeogenic enzymes PEPCK and glucose-6-phosphatase. In fact, the evolutionary conservation of the sequences surrounding Ser9 of GSK-3β has led to the suggestion that two protein kinase cascades, activated by insulin and other growth factors, converge at GSK-3 (14). Although GSK-3 inhibitors may engage alternative targets, glycogen synthase activity was clearly stimulated and was assumed to be the primary mechanism by which GSK-3 inhibition enhanced glucose disposal in the ZDF (*fa/fa*) rat. The ability of these GSK-3 inhibitors to

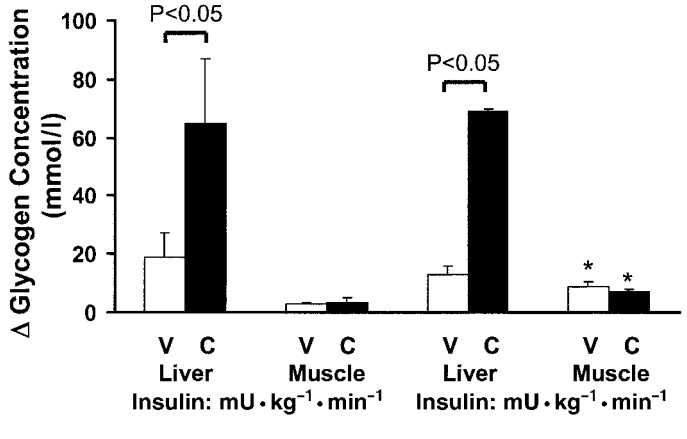


FIG. 5. Change in glycogen concentration in liver and gastrocnemius muscle in ZDF (*fa/fa*) rats treated with vehicle (□) or CHIR99021 (■) after euglycemic-hyperglycemic clamps at insulin infusion rates of 2 or 20 mU · kg^{-1} · min^{-1}. *$P < 0.05$ compared with muscle glycogen concentrations after the 2-mU · kg^{-1} · min^{-1} clamp.

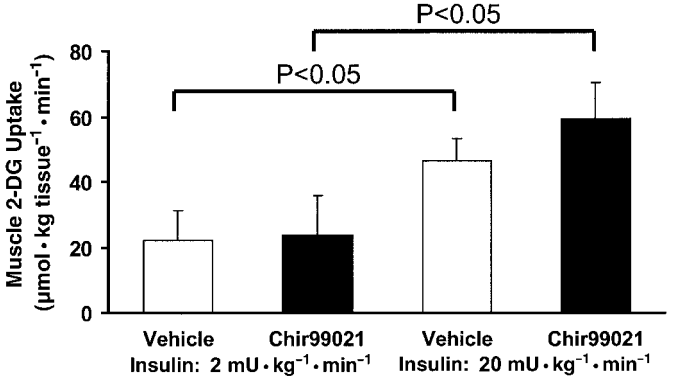


FIG. 6. In vivo rates of 2,6-[^{3}H]-2-deoxy-D-glucose uptake in gastrocnemius muscle in ZDF (*fa/fa*) rats treated with vehicle (□) or CHIR99021 (■) after euglycemic-hyperglycemic clamps at insulin infusion rates of 2 or 20 mU · kg^{-1} · min^{-1}.

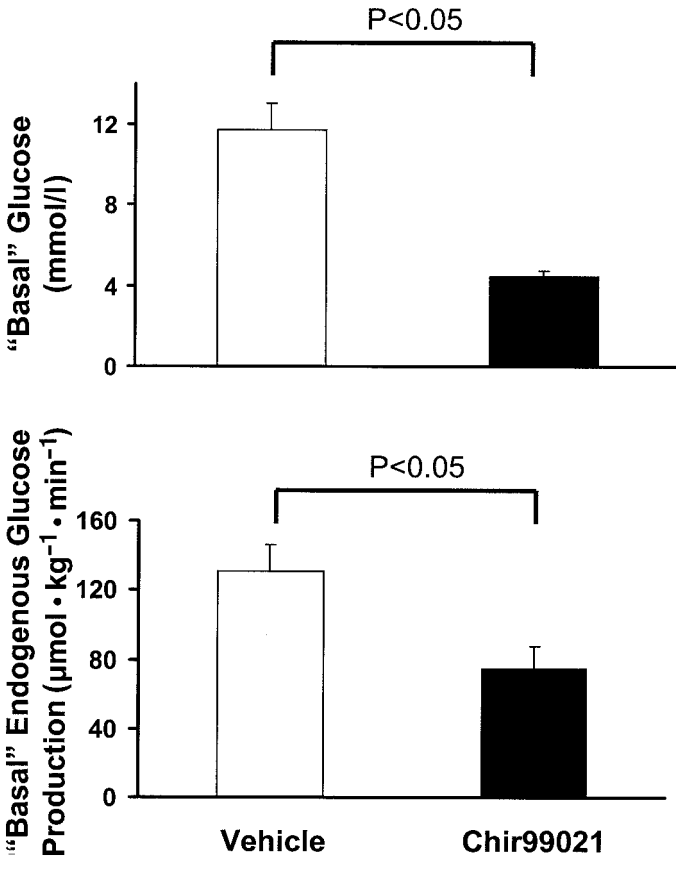


FIG. 7. Fasting blood glucose concentration (*A*) and rates of EGP (*B*) in diabetic ZDF (*fa/fa*) rats after a 20-h infusion of either vehicle (□) or CHIR99021 (■).

significantly enhance glycogen synthase activity, with minimal perturbation of the mechanisms regulating glucose uptake and phosphorylation, provided us with a unique tool to investigate the sites that, we hypothesize, are most important for controlling postprandrial glucose disposal and glycogen synthesis.

Treatment of insulin-resistant ZDF (*fa/fa*) rats with CHIR98023 significantly improved the disposal of an oral glucose load, concomitant with lower levels of plasma insulin (Fig. 2). Similar reductions in hyperglycemia were observed in rats fasted for a shorter 2-h time period that would correspond to the more typical feeding/fasting regimen (data not shown). Inhibition of GSK-3 increased muscle and liver glycogen synthase activity two- to threefold over insulin's effect (Fig. 3). We found that the majority of the enhanced glucose disposal in the CHIR98023-treated rats could be attributed to increased liver glycogen synthesis, with no significant change in the rate of muscle glycogen synthesis (Fig. 4).

To address the possibility that the lower insulin concentrations in the CHIR98023-treated rats during the OGTT masked any beneficial effect of GSK-3 inhibition on muscle glycogen synthesis, we studied the effects of GSK-3 inhibition on insulin-resistant rats during hyperinsulinemic-euglycemic clamps. Using this approach, we examined the effects of GSK-3 inhibitors on *1*) insulin-stimulated rates of liver and muscle glycogen synthesis, *2*) suppression of EGP, and *3*) rates of glucose uptake by skeletal muscle under matched concentrations of plasma insulin and glucose. Under these conditions, we found no effect of GSK-3 inhibition on insulin-stimulated rates of whole-body glucose disposal (Table 1). Whereas previous studies have shown that GSK-3 inhibitors promote glucose uptake into cultured human muscle cells (20) and isolated diabetic rat muscle (21), we did not observe a significant augmentation of insulin-stimulated rates of muscle glucose uptake (Fig. 6) or muscle glycogen synthesis (Fig. 5). In contrast, inhibition of GSK-3 in liver promoted an approximate threefold increase in liver glycogen synthesis (Fig. 5).

TABLE 2
Effect of 20-h preinfusion on basal plasma glucose and insulin concentrations, and glucose infusion rates during euglycemic-hyperinsulinemic clamps in diabetic ZDF (*fa/fa*) rats.

	8 mU · kg^{-1} · min^{-1} insulin infusion rate	
	Control	CHIR99021 treated
Glucose (mmol/l)		
Basal	11.7 ± 1.3	5.8 ± 1.2
Clamp	5.9 ± 0.3	5.1 ± 0.4
Insulin (pmol/l)		
Basal	387 ± 58	319 ± 54
Clamp	1266 ± 316*	776 ± 192*
Glucose infusion rates (μmol · kg^{-1} · min^{-1})		
Clamp	75.0 ± 10.8	72.3 ± 7.8

Data are means ± SE. *$P < 0.05$ compared to basal.

In overtly diabetic rats, overnight exposure to CHIR99021 significantly reduced fasting plasma glucose levels, which could be attributed to a 42% reduction in EGP (Fig. 7). As was seen in the prediabetic insulin-resistant ZDF (*fa/fa*) rats, treatment with GSK-3 inhibitors did not affect the glucose infusion rates needed to maintain euglycemia during the hyperinsulinemic clamp, indicating minimal impact on muscle glycogen synthesis (Table 2).

We did not observe any direct enhancement of glycogen synthesis in skeletal muscle in rats acutely treated with GSK-3 inhibitors. The increase in the rate of skeletal muscle glycogen synthesis correlated with the increase in insulin-stimulated glucose uptake, independent of acute or chronic activation of glycogen synthase by GSK inhibitors. The similar rates of muscle glycogen synthesis in the treated and untreated rats at both insulin levels, despite the threefold increase in muscle glycogen synthase activity, indicate that glycogen synthase activity in skeletal muscle is sufficient to utilize any increased flux into G6P. This result is consistent with previous studies demonstrating that glucose transport is responsible for most of the rate control for insulin-stimulated muscle glucose uptake (2,9–11,36,37). These results provide further evidence that insulin stimulation of skeletal muscle glycogen synthesis operates predominately by a "push" mechanism (38).

Our results indicate that in the insulin-resistant and diabetic ZDF (*fa/fa*) rat, glycogen synthase had minimal control over the rates of glucose metabolism and glycogen synthesis in muscle. Others have argued that the observed increase in skeletal muscle glycogen accumulation in a transgenic mouse model with chronic unregulated glycogen synthase activity supports the hypothesis that defects in glycogen synthesis regulation or activity may contribute to impaired glycogen synthesis in type 2 diabetes (38,39).

However, using the rates of muscle glycogen synthesis that we measured in our OGTT studies, we calculate that a ~5% increase in glycogen synthesis rates over the 4-month life of these mice could lead to the observed two- to fourfold increase of terminal muscle glycogen concentration compared with controls (39). This increase was within the error of our measurement and indicates that long-term cumulative increases in muscle glycogen concentration can occur with minimal control of insulin-stimulated glucose disposal at the level of glycogen synthase.

In contrast to muscle, the rate control of glucose metabolism in liver is distributed among glucokinase and glycogen synthesis, with minimal rate control at the level of glucose transport (40). We have previously shown in humans that insulin inhibits net hepatic glycogenolysis by stimulation of glycogen synthase, with little or no effect on hepatic glycogen phosphorylase, whereas glycogen phosphorylase flux is controlled primarily by plasma glucose concentration, with hyperglycemia leading to inhibition of phosphorylase activity (41). The proportional increase of glycogen synthesis rate with glycogen synthase activity indicates that the locus of rate control is at the level of glycogen synthesis. The similar rates of liver glycogen synthesis at the low and high insulin levels, under the clamp conditions of euglycemia, indicate that both insulin and the GSK-3 inhibitors CHIR98023 and CHIR99021 are targeting the same site in the control of glycogen synthesis. Since CHIR98023 or CHIR99021 activation of glycogen synthase activity was equipotent, regardless of plasma insulin concentration, GSK-3 was most likely maximally inhibited.

In addition to resistance to insulin-stimulated glucose disposal in skeletal muscle, patients with type 2 diabetes have the additional problem that the liver is resistant to insulin's suppressive effect on hepatic glucose output (42–44). The combined effects of impaired glucose uptake and abnormal suppression of EGP contribute to the significantly elevated hyperglycemic excursions after a meal in patients with type 2 diabetes (43,44). Recently, GSK-3 has been shown to be involved in the upregulation of glucose-6-phosphatase and phosphoenolpyruvate gene expression in the liver (22). This has led to the hypothesis that in addition to stimulating glycogen synthesis, selective inhibition of GSK-3 should reduce gluconeogenic flux and hence EGP. In the insulin-resistant rat model acutely infused with CHIR99021, we observed a modest reduction in EGP at the lower insulin infusion rate, perhaps due to decreased gluconeogenic flux. However, since metabolic changes due to changes in gene expression are seen after several hours, the benefits of GSK-3 inhibition of EGP should be more evident after a longer chronic exposure to the drug. An overnight infusion of CHIR99021 lowered fasting plasma glucose in the diabetic ZDF rat by 5.9 ± 1.3 mmol/l ($P = 0.009$), with no significant effect on fasting plasma insulin concentrations (Table 2 and Fig. 7). Because fasting hyperglycemia in the diabetic ZDF rat is predominately due to an increase in EGP, the lower fasting plasma glucose in the 24-h CHIR99021-treated rats is most likely due to the 43% reduction in the rate of EGP (Fig. 7).

OGTT versus glucose-insulin clamps. Metabolism of glucose by liver and muscle is profoundly affected by both circulating levels of insulin and glucose and by the route of glucose administration. In this study, we used two complementary techniques to assess the in vivo effects of GSK-3 inhibition on glucose metabolism. OGTTs provide a means to determine the relative importance of different organs for disposal of a glucose load under physiological conditions, in which the presence of a negative arterial-portal glucose gradient resulting from absorption of glucose by the gut promotes hepatic glucose uptake (45–47). Therefore, an OGTT provides a more relevant measure of the relative importance and magnitude of hepatic glucose disposal under physiological conditions.

In the OGTT, we found a significant increase in liver glycogen synthesis but minimal change in muscle glycogen synthesis, indicating that the liver was primarily responsible for the improvement in glucose disposal in the treated animals. However, since circulating plasma concentrations of glucose and insulin were lower in the treated animal, it was unclear whether treatment with GSK-3 inhibitors had any effect on muscle glucose uptake and metabolism. To address the possibility that GSK-3 inhibition may enhance muscle glucose uptake and metabolism, we used the euglycemic-hyperinsulinemic clamp protocol. As in the OGTT, we saw a negligible effect of drug treatment on rates of muscle glucose uptake or glycogen synthesis. However, since the clamp protocol eliminated the portal glucose gradient, the relative importance of the liver for glucose disposal was masked. Under these conditions, there were no significant differences in rates of EGP. Therefore, differences in the glucose infusion rates needed to maintain euglycemia were primarily due to differences in rates of muscle glucose uptake between the control and CHIR99021-treated rats. Our clamp results therefore corroborate the results from the OGTT studies indicating that the efficacy of GSK-3 inhibition on glucose disposal is mediated primarily by the liver.

In conclusion, Chiron GSK-3-inhibitor treatment resulted in significant activation of glycogen synthase activity in both liver and muscle of the ZDF (*fa/fa*) rat model of type 2 diabetes, which was associated with a significant improvement in oral glucose disposal, which in turn could mostly be attributed to enhanced liver glycogen synthesis. Additionally, long-term Chiron GSK-3-inhibitor treatment significantly lowered fasting plasma glucose in diabetic rats, most likely by reducing EGP. Since both liver glycogen synthesis and regulation of EGP are defective in patients with type 2 diabetes, these data suggest that GSK-3 inhibition may represent an important new therapeutic target for this disease by treating these defects in liver.

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