Acute selective glycogen synthase kinase-3 inhibition enhances insulin signaling in prediabetic insulin-resistant rat skeletal muscle

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tance of glucose transport in the prediabetic state, and the molecular mechanisms involved, have not yet been investigated. Therefore, the present investigation addressed the following specific aims: 1) to assess if selective in vitro inhibition of GSK3 leads to an improvement in insulin action on GS activity and glucose transport activity in isolated skeletal muscle of female insulin-resistant, prediabetic obese Zucker (fa/fa) rat and 2) to determine if these metabolic actions of GSK3 inhibition in prediabetic skeletal muscle are associated with enhanced functionality of selected insulin-signaling elements, including the insulin receptor (IR), IRS-1, phosphatidylinositol 3-kinase (PI 3-kinase), Akt, and GSK3.

METHODS

GSK3 inhibitor. The GSK3 inhibitor CT118637 (kindly provided by Dr. Stephen D. Harrison, Chiron, Emeryville, CA) is structurally very similar to and has identical pharmacokinetic properties to selective GSK inhibitors used previously by our research group (15, 25; reviewed in Ref. 29). It inhibits both GSK3α and GSK3β with $K_i$ values $<$10 nM in an ATP-competitive manner (S. D. Harrison, personal communication). It was $>$95% pure by HPLC.

In the in vitro incubations, the compound was in free base form diluted from a DMSO stock solution. The final DMSO concentration did not exceed 0.5%.

Animals. Female obese Zucker (fa/fa) rats and lean Zucker (Fa/−) 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 obese Zucker rats weighed 300–340 g, whereas the age-matched lean Zucker rats weighed 180–210 g. All animals were housed in a temperature-controlled room (20–22°C) with a 12:12-h light-dark cycle (lights on from 7 AM to 7 PM) at the Central Animal Facility of the University of Arizona. The animals had free access to chow (Teklad, Madison, WI) and water, and all procedures were approved by the University of Arizona Animal Care and Use Committee.

In vitro treatments of skeletal muscle. After overnight food restriction (chow was restricted to 4 g at 5 PM and was consumed immediately), animals were deeply anesthetized at 8 AM 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 flaccid, unmounted state without tension. Each muscle was incubated for 30 min at 37°C in 3 ml of oxygenated (95% $O_2$-5% $CO_2$) Krebs-Henseleit buffer (KHB) with the NaHCO$_3$ concentration set at 14 mM. This KHB was supplemented with 8 mM glucose, 32 mM mannitol, 0.1% BSA (RIA grade; Sigma Chemical), 0.5% DMSO, and the indicated additions of GSK3 inhibitor or insulin. Thereafter, the muscles were either blotted on filter paper, frozen in liquid nitrogen, and used for the determination of GS activity or insulin signaling functionality or were used for assessment of glucose transport activity.

Assessment of GS activity. GS activity was assessed as the activity ratio (activity in the absence of glucose 6-phosphate divided by the activity in the presence of 5 mM glucose 6-phosphate) using the filter paper assay of Thomas et al. (28), as modified by Henriksen et al. (16).

Assessment of glucose transport activity. After the initial 30-min incubation period, the muscles were rinsed for 10 min at 37°C in 3 ml of oxygenated KHB containing 40 mM mannitol, 0.1% BSA, GSK3 inhibitor, and insulin, if present previously. After the rinse period, the muscles were transferred to 2 ml of KHB containing 1 mM 2-deoxy-[1,2-$^3$H]glucose (2-DG, 300 μCi/mmool; Sigma Chemical), 39 mM [U-$^{14}$C]mannitol (0.8 μCi/mmool; ICN Radiochemicals, Irvine, CA), 0.1% BSA, GSK3 inhibitor, and insulin, if previously present. 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 with liquid nitrogen, weighed, and finally placed in 0.5 ml of 0.5 N NaOH. After the muscles were completely solubilized, 5 ml of scintillation cocktail were added, and the specific intracellular accumulation of 2-[1-$^3$H]DG was determined as described previously (14). This method for assessing glucose transport activity in isolated muscle has been validated (12).

Assessment of insulin signaling factor functionality. Muscles were homogenized in 8 vol of ice-cold lysis buffer (50 mM HEPES, 150 mM NaCl, 20 mM sodium pyrophosphate, 20 mM β-glycerophosphate, 10 mM NaF, 2 mM NaN$_3$, 2 mM EDTA, 1% Triton X-100, 10% glycerol, 1 mM MgCl$_2$, 1 mM CaCl$_2$, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.5 μg/ml pepstatin, and 2 mM phenylmethylsulfonyl fluoride). Homogenates were incubated on ice for 20 min and then centrifuged at 13,000 $g$ for 20 min at 4°C. Total protein concentration was determined using the BCA method (Sigma Chemical). For determination of Akt and GSK serine phosphorylation, samples containing equal amounts of total protein were separated by SDS-PAGE on 7.5 or 12% polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA) and transferred to nitrocellulose membranes. Membranes were incubated with antibodies against phospho-Akt Ser$^{473}$ and phospho-GSK3α/β Ser$^{21}$ (Cell Signaling Technology) overnight. In our hands, Ser$^{21}$ phosphorylation of GSK3α in muscle from the obese Zucker rat is very low (unpublished data), and all GSK3 data in this study are restricted to GSK3β Ser$^{21}$ phosphorylation. Subsequently, membranes were incubated with secondary goat anti-rabbit antibody conjugated with horseradish peroxidase (HRP; Chemicon, Temecula, CA). The proteins were visualized on Kodak X-OMAT AR film (Kodak, Rochester, NY) using an enhanced chemiluminescence detection system (Amersham Pharmacia, Piscataway, NJ). The band intensities on the autoradiographs were quantified with the Bio-Rad imaging densitometer (model GS-800) using Quantity One software.

For measurement of tyrosine-phosphorylated IRβ (IR$β$Y) and IRS-1 (IRS-1/pY) and for IRS-1-associated p85 (IRS-1/p85), immunoprecipitations and subsequent immunoblotting were performed. Muscles were homogenized in 1 ml of ice-cold lysis buffer, and total protein concentration was determined as above. Samples were diluted to 2 mg/ml (IRS-1/pY and IR$β$Y) or 1 mg/ml (IRS-1/p85). For assessment of IR$β$Y, 0.5 ml of diluted homogenate was immunoprecipitated with 15 μl of recombinant agarose-conjugated anti-phospho-tyrosine antibody (4G10; Upstate Biotechnology). For analysis of IRS-1/pY and IRS-1/p85, 0.5 ml of diluted homogenate was immunoprecipitated with 25 μl of agarose-conjugated anti-IRS-1 antibody and anti-PI 3-kinase p85 antibody, respectively (Upstate Biotechnology). After an overnight incubation at 4°C, samples were pulse-centrifuged, and the supernatant was removed. The agarose beads were washed three times with ice-cold PBS, mixed with SDS sample buffer, and boiled for 5 min. Equal amounts of the protein of interest were separated by SDS-PAGE on 7.5% polyacrylamide gels and transferred to nitrocellulose membranes. For detection of IR$β$Y and IRS-1/p85, membranes were incubated with the appropriate dilution of commercially available antibodies against IR $β$-subunit (for IR$β$Y) and IRS-1 (for IRS-1/p85; Upstate Biotechnology). For analysis of IRS-1/pY, the nitrocellulose membrane was incubated in anti-phosphotyrosine antibody (PY99; Santa Cruz Biotechnology, Santa Cruz, CA). Thereafter, the membranes were incubated with secondary goat anti-mouse antibody conjugated with HRP (Santa Cruz Biotechnology). Protein bands of interest were exposed, visualized, and quantified as described above.

Statistical analysis. All values are expressed as means ± SE. All experiments were done with paired muscles from the same animal incubated with or without the GSK3 inhibitor. Therefore, differences between the two groups resulting from the GSK3 inhibitor alone (in the absence or presence of insulin) were analyzed using a paired Student’s t-test. A level of $P < 0.05$ was set for statistical significance.
RESULTS

Phosphorylation state of GSK3 in lean and obese muscle. The degree of GSK3 Ser\(^9\) phosphorylation, inversely related to GSK3 activity, was assessed in the basal and insulin-stimulated states in epitrochlearis and soleus muscles from lean and obese Zucker rats (Fig. 1). Basal GSK3\(^\beta\) phosphorylation was not different in the epitrochlearis between lean and obese groups, whereas this variable was 46% less (P < 0.05) in obese soleus compared with the lean soleus. In the insulin-stimulated state, GSK3\(^\beta\) phosphorylation was less in both the obese epitrochlearis (14%) and the obese soleus (23%) relative to the respective lean groups, indicating a greater GSK3\(^\beta\) activity in the insulin-stimulated condition. These data are in agreement with previous studies demonstrating a greater GSK3 activity in insulin-resistant skeletal muscle from obese type 2 diabetic rats (2) and humans (22). Protein expression of GSK3\(^\beta\) in epitrochlearis and soleus was not different between lean and obese groups (data not shown).

Effect of selective GSK3 inhibition on GS activity. To establish the effectiveness of CT118637 to inhibit GSK3 in vitro, we assessed the modulation of the GS activity ratio in the absence or presence of the GSK3 inhibitor in skeletal muscle from lean and obese Zucker rats (Fig. 2). In the lean Zucker rats, 1 \(\mu\)M CT118637 enhanced (P < 0.05) basal GS activity in epitrochlearis (88%) and soleus (106%) muscles (Fig. 2A). A maximally effective concentration of insulin (5 \(mU/ml\)) increased GS activity by 56% in lean epitrochlearis and by 57% in lean soleus. The combination of the GSK3 inhibitor and insulin increased GS activity above basal by 154% in lean epitrochlearis and by 138% in lean soleus. The effects of CT118637 and insulin in combination on GS activation in muscles from lean Zucker rats were essentially additive. The interventions did not alter the total activity of GS in these muscles (data not shown).

In obese Zucker rats, 1 \(\mu\)M CT11837 enhanced (P < 0.05) basal GS activity in both epitrochlearis (81%) and soleus (108%) muscles (Fig. 2B). These increases were far greater than those observed with insulin alone, which increased GS activity by 30% in obese epitrochlearis and by 31% in obese soleus muscles. The combination of the GSK3 inhibitor and insulin increased GS activity by 133% in obese epitrochlearis and by 144% in obese soleus. Notably, the GSK3 inhibitor increased insulin-stimulated GS activity in a synergistic fashion in muscle from the obese Zucker rats, similar to our previous observations in muscle from ZDF rats (15, 25).

Effect of selective GSK3 inhibition on glucose transport activity. In both epitrochlearis and soleus muscles from the lean Zucker rats, GSK3 inhibition did not alter either basal or insulin-stimulated glucose transport activity (Fig. 3A). Similarly, selective in vitro GSK3 inhibition had no effect on basal glucose transport activity in epitrochlearis or soleus muscles from the obese Zucker rats (Fig. 3B). In contrast, in vitro treatment with the selective GSK3 inhibitor CT118637 enhanced (P < 0.05) insulin-stimulated glucose transport activity in epitrochlearis (24%) and soleus (20%) muscles of the obese Zucker rat. The absolute effect of GSK3 inhibition to enhance insulin-stimulated glucose transport activity was nearly two
times as great in the obes type I soleus (87 ± 12 pmol·mg⁻¹·20 min⁻¹) as it was in the obes type IIb epitrochlearis (45 ± 12). Therefore, as shown previously with the selective GSK3 inhibitor CT98014 in skeletal muscle from overtly type 2 diabetic ZDF rats (15, 25), the beneficial effect of GSK3 inhibition on glucose transport activity is restricted to insulin-resistant skeletal muscle and is greater in oxidative muscle than in glycolytic muscle.

Effects of selective GSK3 inhibition on insulin signaling. To identify a potential molecular mechanism responsible for the enhanced insulin-stimulated glucose transport activity in obes skeletal muscle due to GSK3 inhibition (Fig. 3B), the functionality of important elements of the insulin signaling cascade were assessed (Figs. 4–8). GSK3 inhibition did not affect either basal or insulin-stimulated tyrosine phosphorylation of the IR β-subunit in obes epitrochlearis and soleus muscles (Fig. 4). In contrast, significant increases (P < 0.05) in the level of IRS-1 tyrosine phosphorylation were observed in insulin-stimulated obes epitrochlearis (40%) and soleus (45%) muscles in the presence of the GSK3 inhibitor (Fig. 5). Basal IRS-1 tyrosine phosphorylation was not affected by the GSK3 inhibition.

The functionality of additional downstream elements of the insulin signaling cascade, including PI 3-kinase, Akt, and GSK3, was assessed. No effects of GSK3 inhibition in the absence of insulin were observed for these factors (Fig. 6–8). However, in the obes soleus, GSK3 inhibition in the presence of insulin induced significant enhancements of IRS-1 associated with the p85 subunit of PI 3-kinase (a surrogate measure of PI 3-kinase activity, 72%), Ser⁴⁷³ phosphorylation of Akt1/2 (30%), and Ser⁹ phosphorylation of GSK3β (39%). GSK3 inhibition also increased the insulin stimulation of these factors in the obes epitrochlearis muscle, but these effects were less (44, 21, and 13%, respectively) than in the obes soleus muscle.
DISCUSSION

We have demonstrated in the present investigation that acute in vitro treatment of isolated skeletal muscle from insulin-resistant, prediabetic female obese Zucker rats with the selective GSK3 inhibitor CT118637 (1 μM) can potentiate the ability of insulin to stimulate both GS activity (Fig. 2) and glucose transport activity (Fig. 3). Importantly, although GSK3 inhibition was manifested in skeletal muscle from both lean and obese animals, as evidenced by increases in the GS activity ratio (Fig. 2), insulin action on glucose transport activity was enhanced only in insulin-resistant obese muscles, and selective inhibition of GSK3 had no effect on glucose transport activity in insulin-sensitive skeletal muscles from lean Zucker rats (Fig. 2). These results are consistent with previous studies showing that selective inhibition of GSK3 enhances insulin-stimulated glucose transport only in insulin-resistant tissues of type 2 diabetic rats (15, 24) and not in insulin-sensitive tissues (15, 18, 25). Moreover, these data support the hypothesis that elevated GSK3 activity, which we have shown here, exists in muscle from the obese Zucker rat (Fig. 1) and has been reported previously to be present in muscle of insulin-resistant, obese rats (2) and humans with type 2 diabetes (22), is necessary for modulation of glucose transport activity by GSK3 inhibition.

An additional novel finding of the present investigation is that the increased insulin-stimulated glucose transport activity in response to in vitro GSK inhibition in the skeletal muscle from the obese Zucker rat was associated with an enhancement of insulin-stimulated IRS-1 tyrosine phosphorylation (Fig. 5), a critical element of the insulin signaling cascade in muscle (32). However, it is important to note that the enhancement of insulin action mediated by the GSK3 inhibitor was clearly distal to the IR in obese skeletal muscle, since a similar GSK3 inhibitor-mediated upregulation of insulin action was not observed for tyrosine phosphorylation of the IR β-subunit (Fig. 4). These results are in agreement with and complement the findings of Eldar-Finkelman and Krebs (9), who demonstrated that overexpression of GSK3 diminishes normal insulin signaling by phosphorylating IRS-1 on serine residues, which in turn attenuates the ability of IRS-1 to undergo tyrosine phosphorylation catalyzed by IR.

The enhanced insulin-stimulated IRS-1 tyrosine phosphorylation induced by the GSK3 inhibitor appears to have important downstream effects in the insulin signaling cascade, since insulin action on IRS-1 associated with the p85 subunit of PI 3-kinase (Fig. 6), Ser473 phosphorylation on Akt (Fig. 7), and Ser^β phosphorylation of GSK3β (Fig. 8) were similarly upregulated in skeletal muscle from the obese Zucker rat incubated with the selective GSK3 inhibitor. Moreover, the enhancement of IRS-1 associated with p85 elicited by the GSK3 inhibition is likely causally related to the increase in insulin-stimulated glucose transport activity, since the activation of PI 3-kinase by tyrosine-phosphorylated IRS-1 is necessary for insulin-stimulated translocation of GLUT4 protein to the cell surface (32). Although GLUT4 protein translocation was not assessed in the present study, we have previously reported that in vitro GSK3 inhibition with a compound of similar molecular structure and action causes enhanced insulin-stimulated cell-surface GLUT4 in skeletal muscle from insulin-resistant skeletal muscle from ZDF rats (15).

These new data also support a mechanistic connection between the upregulation of Akt serine phosphorylation (Fig. 7) and GSK3β serine phosphorylation (Fig. 8) and the potentiation of insulin stimulation of GS activity (Fig. 2) resulting from GSK3 inhibition. The enhanced insulin-stimulated Akt activity (as reflected by the increased serine phosphorylation state) would catalyze greater GSK3 phosphorylation and lead to less GSK3 activity, thereby further decreasing the ability of GSK3 to phosphorylate and inactivate GS, a direct target of GSK3 (6). Both the direct inhibitory effect of CT118637 on GSK3 and the reduced GSK3 activity resulting from covalent modifications of the enzyme resulting from enhanced Akt

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Fig. 6. Effect of GSK3 inhibition on basal or insulin-stimulated IRS-1 associated with p85 subunit of phosphatidylinositol 3-kinase (PI 3-kinase) in skeletal muscle of obese Zucker rats. Epitrochlearis and soleus strips were incubated without or with CT118637 (1 μM) in the absence or presence of insulin (5 μU/ml) for 30 min. Assessment of IRS-1 associated with the p85 subunit of PI 3-kinase was then performed as described in Methods. For each muscle type, the insulin-stimulated value in the absence of GSK3 inhibitor was arbitrarily set at 100%. Representative bands are shown. Values are means ± SE for 5 muscles/group. *P < 0.05 vs. paired muscles in the absence of GSK3 inhibitor.

Fig. 7. Effect of GSK3 inhibition on basal or insulin-stimulated Akt Ser473 phosphorylation in skeletal muscle of obese Zucker rats. Epitrochlearis and soleus strips were incubated without or with CT118637 (1 μM) in the absence or presence of insulin (5 μU/ml) for 30 min. Assessment of the phosphorylation of Ser^β on Akt1/2 was then performed as described in Methods. For each muscle type, the insulin-stimulated value in the absence of GSK3 inhibitor was arbitrarily set at 100%. Representative bands are shown. Values are means ± SE for 5–7 muscles/group. *P < 0.05 vs. paired muscles in the absence of GSK3 inhibitor.
functionality can account for the increased insulin-stimulated activation of GS in the presence of the GSK3 inhibitor CT118637. In addition, an increased ability of insulin to activate the muscle-specific glycogen-associated type I protein phosphatase, which catalyzes the dephosphorylation and activation of GS (reviewed in Ref. 20), may also have contributed to the greater enhancement of insulin-dependent GS activity with the GSK3 inhibitor. Collectively, these findings underscore the important physiological role of GSK3 in the regulation of GS activity in insulin-resistant skeletal muscle.

It is noteworthy that the effects of the GSK3 inhibitor CT118637 to enhance insulin action on glucose transport activity and on post-IR insulin signaling were quantitatively greater in the type I soleus muscle than in the type IIb epitrochlearis muscle of the obese Zucker rat. We have observed similar fiber type-specific effects of selective GSK3 inhibition on glucose transport activity in skeletal muscle of the type 2 diabetic ZDF rat (15). One factor that may underlie this differential response to GSK3 inhibition is the greater absolute degree of insulin resistance of glucose transport activation in the soleus compared with the epitrochlearis of these obese Zucker rats (Fig. 3). This greater degree of insulin resistance in the obese soleus is also reflected in a lesser insulin-stimulated GSK3β phosphorylation (relative to the respective insulin-stimulated lean muscle) in the obese soleus compared with the obese epitrochlearis (Fig. 1). This reflects a greater GSK3 activity in the obese soleus compared with the obese epitrochlearis and could perhaps make the obese soleus more susceptible to metabolic modification by a GSK3 inhibitor. An additional factor in this differential response to the GSK3 inhibitor is that the protein expression and/or functionality of the insulin signaling factors of interest in the present study, including IRS-1 tyrosine phosphorylation, phosphorytrosine-associated PI 3-kinase activity, and Akt Ser173 phosphorylation, are greater in type I muscle than in type IIb muscle (26). In addition, the protein expression of GLUT4 is likewise greater in type I soleus muscle than in type IIb epitrochlearis muscle (13). Taken together, it is clear that type I muscle has a greater capacity for insulin signal transduction and activation of glucose transport than does type IIb muscle; therefore, one would expect that an intervention, such as selective GSK3 inhibitors, that acts on the functionality of the insulin signaling elements would invoke a more robust response in type I muscle compared with type IIb muscle.

Although the present investigation was restricted to the in vitro application of a selective GSK3 inhibitor, our research group and others have recently demonstrated that improvements in whole body glucose disposal can be elicited when selective GSK3 inhibitors are administered systemically to insulin-resistant obese animal models (15, 24, 25). The disposal of an oral glucose load is significantly enhanced in ZDF rats treated with structurally and functionally similar GSK3 inhibitors (CT98023 and CT99021; see Refs. 15 and 25). These improvements in glucose tolerance were realized without a corresponding increase in the endogenous insulin level, indicating a reduction in insulin resistance (15, 25). Similar findings have been found in other insulin-resistant obese animal models, including the ob/ob mouse, the db/db mouse, and the high-fat-fed rat (25). In addition, intraperitoneal administration of a novel peptide-based GSK3 inhibitor, which acts at the catalytic site of the enzyme, causes improved glucose tolerance in the insulin-resistant obese C57BL/6J mouse (24). Moreover, the recent finding by Pearce and colleagues (23) that selective overexpression of GSK3β in skeletal muscle of male mice is associated with a marked diminution of glucose tolerance and with a reduced development of hyperinsulinemia and dyslipidemia further underscores the negative influence of muscle GSK3 on glucoregulation. These various in vivo results indicate that the development of GSK3 inhibitors for modulation of insulin action is a rational approach for the treatment of insulin resistance and type 2 diabetes.

Our demonstration of the effectiveness of the selective GSK3 inhibitor to enhance insulin action in the prediabetic obese Zucker rat is an important extension of previous studies showing an increase in insulin action in muscle of overtly diabetic ZDF rats (15, 25). The prediabetic state is characterized by insulin resistance and marked hyperinsulinemia, but not by fasting hyperglycemia. This is a particularly relevant state of metabolic dysfunction, since individuals with prediabetes have an elevated risk for the development of type 2 diabetes (11, 30). An important conclusion from the present investigation is that these GSK3 inhibitors are also effective in muscle that has not previously been exposed to marked hyperglycemia in vivo.

In summary, selective in vitro GSK3 inhibition with CT118637 increased insulin stimulation of glucose transport activity in insulin-resistant skeletal muscle of prediabetic, obese Zucker rats but not in insulin-sensitive skeletal muscle of lean Zucker rats. The GSK3-induced improvement of insulin action in the insulin-resistant, prediabetic skeletal muscle was associated with enhancement of IRS-1 tyrosine phosphorylation, IRS-1 associated with the p85 subunit of PI 3-kinase, Akt Ser173 phosphorylation, and GSK3β Ser48 phosphorylation but not with IR tyrosine phosphorylation. Finally, the beneficial effects of GSK3 inhibition on insulin action were generally greater in type I muscle (soleus) than in type IIb muscle (epitrochlearis) from these insulin-resistant, prediabetic obese animals. Collectively, these results provide support for selec-
tively targeting GSK3 in the treatment of skeletal muscle insulin resistance in the prediabetic state.

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