THE ROLE OF GLYCOGEN SYNTHASE KINASE-3 IN INSULIN-RESISTANT SKELETAL MUSCLE

By

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ABSTRACT

The metabolic syndrome is a multifaceted condition characterized by a clustering of metabolic and cardiovascular abnormalities, including insulin resistance in skeletal muscle, adipose tissue and liver, visceral adiposity, hyperinsulinemia, glucose intolerance, dyslipidemia, and essential hypertension. Those affected by this syndrome are at very high risk for developing type 2 diabetes and all of the related sequelae.

Glycogen synthase kinase-3 (GSK-3) is a serine/threonine kinase that contributes to the multi-factorial etiology of insulin resistance by attenuating insulin signaling in skeletal muscle, thereby decreasing glucose uptake. GSK 3 is overactive in humans with type 2 diabetes, and in animal models of both type 2 diabetes and the metabolic syndrome (pre-diabetes). Selective GSK-3 inhibition reversed several facets of insulin resistance in the obese Zucker (fa/fa) rat, a model of pre-diabetes and the metabolic syndrome. Acute GSK-3 inhibition in skeletal muscle improved insulin-stimulated glucose uptake and glycogen synthase activity, and enhanced the functionality of key components of the insulin signaling pathway. In addition, GSK-3-β activity was decreased. Chronic selective GSK-3 inhibition improved whole-body insulin-sensitivity, reduced plasma free fatty acids, increased insulin-stimulated glucose uptake into isolated skeletal muscle, and enhanced insulin signaling in skeletal muscle.

Oxidative stress is another etiologic component of insulin resistance, and type 2 diabetes is associated with higher levels of oxidant stress. Oxidant stress was induced in isolated muscle of insulin-sensitive lean Zucker rats, a model of normal glucose

metabolism. Oxidant stress reduced insulin-stimulated glucose transport, glycogen synthesis, and glycogen synthase activity by ~50%, and reduced the ability of insulin to de-activate GSK-3ß. In the presence of oxidant stress, the GSK-3 inhibitor improved insulin-stimulated glucose transport, insulin stimulated glycogen synthesis, glycogen synthase activity and insulin signaling. Selective GSK-3 inhibition, therefore, partially ameliorated the skeletal muscle insulin resistance caused by oxidative stress.

The results of the current study suggest that GSK-3 overactivity contributes to the multi-factorial etiology of obesity-associated insulin resistance as well as insulin resistance related to oxidative stress. Taken together, these findings support the potential of selective GSK-3 inhibition to ameliorate, in part, the insulin resistance associated with the metabolic syndrome and type 2 diabetes, and worsened by oxidative stress.

CHAPTER 1

INTRODUCTION AND BACKGROUND

Type 2 diabetes is an epidemic both domestically and internationally, currently affecting upwards of 20 million individuals in the United States [17], and it is predicted that the number of people with type 2 diabetes world-wide will increase to over 300 million by the year 2025 [61]. The pre-diabetic state is defined as the presence of fasting plasma glucose levels between 100 and 125 mg/dl that do not exceed the criteria ($\geq$126 mg/dl) for diagnosis of diabetes [3], and immediately precedes the development of overt type 2 diabetes [10]. It is estimated that over 40 million people in the United States have pre-diabetes [3]. Insulin resistance of skeletal muscle glucose transport and metabolism is a hallmark of both the pre-diabetic state and overt type 2 diabetes [48;124]. The etiology of skeletal muscle insulin resistance is clearly multi-factorial and can involve defective expression and functionality of multiple elements in the insulin signaling cascade that regulates the glucose transport process (see reviews in [48;124].

One factor that has been implicated in the etiology of insulin resistance is the enzyme glycogen synthase kinase-3 (GSK-3), which is known to be over-active in insulin-resistant tissues. Studies conducted in our lab and others have demonstrated that the selective inhibition of GSK-3 mitigates insulin resistance in a variety of cell models, animal models, and human tissues; however, most of these experiments have been conducted *in vitro*; and the few *in vivo* studies that have been done have used acute rather than prolonged treatments. Moreover, no previous studies have investigated the effects of selective GSK-3 inhibition in a pre-diabetic animal mode of obesity-associated insulin resistance. In addition, the precise molecular mechanisms associated with the effects of selective GSK-3 inhibition have not been elucidated

The condition of oxidative stress, which occurs when the production of reactive oxygen species (ROS) exceeds the anti-oxidant defense capabilities of a tissue, also contributes to insulin-resistance. Obesity alone, insulin-resistance, and type 2 diabetes are all conditions associated with elevated levels of oxidative stress, purportedly related to the excessive metabolism of glucose and free fatty acids present in these physiologic states. In addition, oxidative stress can lead to mitochondrial dysfunction and subsequent defects in oxidative phosphorylation, which can, in turn, further increase ROS production [67].

The ROS-associated insulin-resistant state has been created experimentally in lean, insulin-sensitive skeletal muscle *in vitro*, and these studies have demonstrated diminished glucose transport associated with defects in the insulin signaling pathway, including an impaired ability of insulin to stimulate the phosphorylation and de-activation of GSK-3 [97]. Thus, it is possible that the condition of oxidative stress may contribute to the over-activity of GSK-3, and that the presence of both conditions together might cause greater insulin resistance than either alone. Consequently, selective inhibition of GSK-3 under the conditions of oxidative stress may mitigate the related defects in insulin action.

The major goals of this study are to further clarify the role of GSK-3 in obesity-associated insulin resistance and pre-diabetes, and to investigate the potential role of GSK-3 in the insulin-resistant state induced by oxidative stress. The obese Zucker (fa/fa) rat will be used as the model for obesity-associated insulin resistance and pre-diabetes experiments. The lean Zucker rat will be used as the model for insulin-sensitive skeletal

muscle, and these muscles will be exposed to an oxidant stress to induce insulin resistance in vitro.

Regulation of skeletal muscle glucose transport

Regulation of whole-body glucose homeostasis is a function of the production of glucose from the liver and of the peripheral disposal of glucose, primarily in skeletal muscle. These two physiological processes are under the influence of several endocrine factors, the most important of which is insulin, secreted by the ß-cells of the pancreatic islets. In order to appreciate the significance of defects in glucose control that characterize the pre-diabetic and diabetic states, a brief description of the normal functionality of these glucoregulatory processes is warranted.

Skeletal muscle comprises over 40% of the body mass of humans and other mammalian species and is the major tissue responsible for the peripheral disposal of glucose in response to a glucose or insulin stimulus or during endurance exercise bout [7;27]. Skeletal muscle glucose transport activity is acutely regulated by insulin through the activation of a series of intracellular proteins (for reviews, see [48;99;113;124]. Insulin binding to the α-subunit of the insulin receptor stimulates tyrosine kinase activity of the membrane-spanning ß-subunits, leading to autophosphorylation of these ß-subunits and enhancing the intrinsic tyrosine kinase activity. The insulin receptor then phosphorylates insulin receptor substrates (primarily IRS-1 in skeletal muscle) at conserved pYXXM sequences, thereby allowing the tyrosine-phosphorylated IRS-1 to dock with the SH2 domains of the p85 regulatory subunit of phosphotidylinositol-3-kinase (PI3-kinase). This interaction activates the p110 catalytic subunit of PI3-kinase,

which catalyzes the production of phosphoinositide moieties that subsequently activate 3-phosphoinositide-dependent kinases (PDKs), including PDK1. One downstream target of PDK1 is the serine/threonine kinase Akt, and the sequential activation of these steps ultimately results in the translocation of a specific glucose transporter protein isoform (GLUT-4) to the membranes of the sarcolemma and the t-tubules, where glucose transport takes place via a facilitative diffusion process. The amount of GLUT-4 protein incorporated into the sarcolemmal membrane correlates closely with the degree of insulin-stimulated glucose transport, and strongly suggests that GLUT-4 translocation represents the major mechanism for insulin-stimulated glucose transport in skeletal muscle of rodents [33;38;71] and humans [42].

Regulation of Hepatic Glucose Production. Glucose production by the liver is mediated by both the breakdown of glycogen stores (glycogenolysis) and the synthesis of new glucose molecules (gluconeogenesis) from a variety of precursor molecules delivered to the liver. The regulation of these processes is under exquisite hormonal control, primarily by the actions of insulin and glucagon, with additional longer-term effects mediated by epinephrine and cortisol (reviewed in [16;111]). Under conditions of increased glucose demand by peripheral tissues, such as by contracting skeletal muscle during moderate-intensity exercise, hepatic glucose production must be enhanced accordingly or overt hypoglycemia can quickly develop. In uncompromised individuals (i.e., non-diabetics), the most important hormonal adjustments that augment hepatic glucose production during exercise is a decrease in insulin secretion and an increase in glucagon secretion, with increases in epinephrine and cortisol playing important

secondary roles with more prolonged (>1 hr) bouts of endurance exercise [111]. These endocrine adjustments are brought about by acute autonomic neural inputs to the endocrine glands, possibly as a result of signals derived from the contracting muscle, from neural activity originating in the splanchnic bed, subtle changes in glucose availability, and neural input from the motor centers [112].

Hepatic gluconeogenesis is under the control of the gene expression of at least two highly-regulated enzymes, phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase). Insulin is known to suppress the expression of PEPCK and G6Pase, while glucagon and glucocorticoids enhance the expression of these genes [5;45;82;103]. The enhancement of hepatic gluconeogenesis is vital for the maintenance of hepatic glucose production under conditions of longer-term increases in peripheral glucose utilization, such as prolonged moderate-intensity exercise [111].

Disruption of Whole-body Glucose Regulation. The two major causes of diabetes are insulin deficiency (types 1 and 2) and insulin resistance (type 2). In both cases, insulin action is defective and plasma glucose levels become elevated. Insulin resistance occurs in skeletal muscle, adipose tissue and the liver. Skeletal muscle is responsible for the majority of glucose disposal; adipose tissue plays a minor role and, like skeletal muscle, requires GLUT-4 translocation for the facilitated diffusion of glucose intracellularly. Insulin resistance in the liver manifests as excess hepatic glucose output; most likely a result of impaired insulin signaling to enzymes involved in its regulation.

Etiology of Skeletal Muscle Insulin Resistance. Insulin resistance of skeletal muscle glucose transport represents a major defect in the normal maintenance of

euglycemia [48;124] and is often accompanied by a variety of metabolic abnormalities, including hypertension, type 2 diabetes mellitus or pre-diabetes, dyslipidemia, atherosclerosis, and central adiposity, a condition referred to collectively as the metabolic syndrome [41]. A strong link exists between these metabolic abnormalities and cardiovascular morbidity and mortality. Humans with the metabolic syndrome but normal plasma glucose levels are at high risk for cardiovascular events; as glycemic control worsens, cardiovascular risk increases, and overt type 2 diabetes is one of the strongest known risk factors for cardiovascular disease [114]. Type 2 diabetes is a progressive disease involving a β-cell defect that worsens over time, and occurs when increased insulin production (hyperinsulinemia) can no longer compensate for the high demand caused by insulin resistance and plasma glucose levels rise [108]. Individuals with pre-diabetes who intervene (with medications or exercise and weight loss) to improve their insulin sensitivity can decrease their risk of developing overt type 2 diabetes by 38-58% [28]. Improvements in insulin action on skeletal muscle glucose metabolism in insulin-resistant individuals are therefore expected to decrease conversion rates to overt diabetes, as well as to reduce cardiovascular morbidity and mortality.

Several studies have identified skeletal muscle as the primary cellular locus for the insulin resistance of glucose disposal associated with obesity in both animal models and humans. One set of investigations has used the obese Zucker (*fa/fa*) rat, a genetic model of severe skeletal muscle insulin resistance that is also characterized by marked hyperinsulinemia, glucose intolerance (pre-diabetes), dyslipidemia, and central adiposity [76]. Insulin-stimulated GLUT-4 protein translocation [33;65;76] and glucose transport

activity [23;33;52] are substantially impaired in isolated skeletal muscle from these obese animals. The insulin resistance characteristic of the obese Zucker rat can be attributed to specific defects in the insulin signaling pathway for activation of glucose transport. Compared to age-matched, insulin-sensitive lean Zucker rats, skeletal muscle from obese Zucker rats displays reductions in insulin-stimulated insulin receptor tyrosine phosphorylation [97] and in IRS-1 protein level and insulin-stimulated IRS-1 phosphorylation [4;56;97]. The amount of the p85 regulatory subunit of PI3-kinase associated with the tyrosine-phosphorylated IRS-1 in the insulin-stimulated state and IRS-1-associated PI3-kinase activity in muscle from these obese animals are both defective [4;97].

Similar metabolic defects in skeletal muscle have been observed in insulin-resistant human subjects, the vast majority of whom are obese. Zierath et al. [123] have reported that insulin stimulation in vivo failed to induce significant GLUT-4 protein translocation to the sarcolemma in muscle from subjects with type 2 diabetes. Moreover, Goodyear et al. [40], using isolated strips of rectus abdominis muscle from obese, insulin-resistant subjects, and Björnholm et al. [12], using muscle biopsies from lean to moderately obese type 2 diabetic patients, have demonstrated that significantly less insulin stimulation of insulin receptor and IRS-1 tyrosine phosphorylation and of IRS-1-immunoprecipitatable PI3-kinase activity is detected in insulin-resistant muscle of the diabetic subjects. It has also been demonstrated that insulin-stimulated Akt kinase activity is significantly reduced in skeletal muscle from type 2 diabetic subjects [68].

Hepatic Glucose Overproduction. Elevations in glucose production by the liver are thought to contribute to the dysregulation of glucose homeostasis in type 2 diabetic humans [90]. Several factors may be associated with this excess hepatic glucose production. In obese type 2 diabetic subjects, insulin action on hepatic glycogen synthase is reduced [19], there is a lesser ability of insulin to suppress hepatic glucose production (likely via a decreased inhibition of hepatic glycogenolysis) [72], and the rate of hepatic gluconeogenesis is elevated [73]. In addition, the relative decrease in insulin secretion by the pancreatic ß-cells, which is an obligatory event in the conversion from the pre-diabetic condition to a state of overt type 2 diabetes, will also contribute to this enhancement of hepatic glucose production [90].

Role of GSK 3 in the etiology of glucose dysregulation.

GSK-3 is a serine/threonine kinase that consists of highly homologous α and ß isoforms [117]. Among its various functions, this enzyme serves to phosphorylate and inactivate glycogen synthase [84;92;122]. GSK 3 is constitutively active in cells, and can be acutely de-activated by insulin signaling through the sequential activation of IRS-1, PI3-kinase, and ultimately via the action of Akt to phosphorylate specific serine residues on the enzyme [24]. The very similar time courses for the insulin-dependent activation of Akt and inactivation of GSK-3 is consistent with the concept that GSK-3 is a physiologically relevant substrate for Akt [115]. An additional substrate of GSK-3 is IRS-1, and phosphorylation of IRS-1 on serine and threonine residues leads to impairment of insulin signaling [31]. These observations support the hypothesis that

GSK-3 can serve as a negative modulator of insulin action on glycogen synthase and, potentially, on glucose transport activity (fig 1).

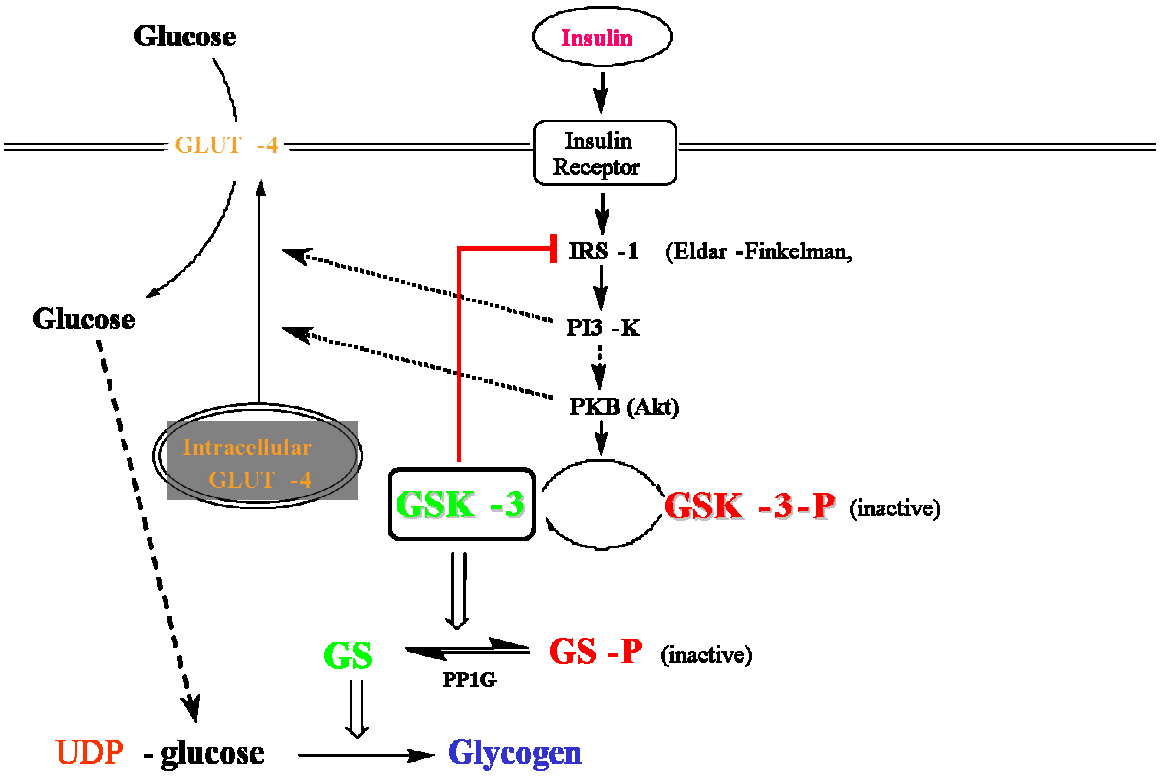


FIGURE 1. The deleterious effects of GSK-3 on key steps in the insulin signaling pathway.

While the etiology of skeletal muscle insulin resistance is multifactorial, recent evidence supports a role of elevated GSK-3 activity as a contributing factor in this pathophysiological state. GSK-3 activity is elevated in tissues of insulin-resistant obese rodent models, including high fat-fed mice [32] and the Zucker Diabetic Fatty rat, a model of type 2 diabetes [15]. In addition, GSK-3 is enhanced in skeletal muscle of obese humans [15;54] and type 2 diabetic humans [78]. The elevation in GSK-3 protein

in skeletal muscle of type 2 diabetic subjects is negatively correlated with both insulin-stimulated skeletal muscle glycogen synthase activity and whole-body glucose disposal [78]. Collectively, these findings are consistent with GSK-3 being a molecule that can indirectly modulate glucose transport and metabolism in skeletal muscle.

An important finding regarding the molecular basis of insulin resistance is the observation that serine phosphorylation of IRS-1 near the phosphotyrosine binding site (ser^{307} in rat IRS-1) is associated with an impairment of tyrosine phosphorylation of IRS-1 by the insulin receptor and with a decrease in PI3-kinase activity [1]. As GSK-3 is known to phosphorylate IRS-1 on serine residues [31], the possibility exists that GSK-3 mediates its effect to decrease insulin signaling in skeletal muscle by phosphorylating ser^{307} or another serine residue that serves as a negative modulator of IRS-1.

The effect of GSK-3 overexpression on whole-body glucose tolerance in mice has been recently investigated [85]. These investigators produced a mouse model that selectively overexpresses the GSK-3ß isoform in skeletal muscle by 5-fold. Relative to non-transgenic control mice, male GSK-3ß transgenic mice are characterized by increased fat mass, decreased muscle protein expression of IRS-1, and decreased glycogen synthase activity and glycogen levels in muscle. Moreover, in response to an oral glucose challenge, the male GSK-3ß transgenic mouse displays an exaggerated glucose response and elevated insulin levels, indicating a decrease in whole-body insulin sensitivity. These male GSK-3ß transgenic mice also show evidence of dyslipidemia, as both fasting plasma levels of free fatty acids and triglycerides are elevated [85].

GSK-3 and Hepatic Glucose Overproduction. GSK-3 can phosphorylate glycogen synthase on multiple serine residues and inactivate this enzyme, thereby reducing glycogenesis [25;102]. This will indirectly potentiate hepatic glucose production derived from glycogenolysis. There is also indirect evidence for a role of GSK-3 in hepatic gluconeogenesis from in vitro studies indicating that GSK-3 can phosphorylate (on ser^{129}) and activate cAMP-responsive element binding (CREB) protein [35]. The activated CREB could then activate PEPCK expression [120] and thereby upregulate the rate of hepatic gluconeogenesis. These data suggest that the over-activity of GSK-3 may contribute to the excess hepatic glucose output observed in type 2 diabetes. However, selective in vitro inhibition of GSK-3 can repress promoter activity of the PEPCK and G6Pase genes [70], which could reduce the rate of gluconeogenesis. In light of these conflicting results, more definitive experiments are needed to support a direct role of GSK-3 in hepatic glucose production.

Role of reactive oxygen species (ROS) in the etiology of glucose dysregulation.

Oxidative damage of proteins is one of the modifications leading to severe failure of biological functions and cell death. Prolonged exposure of protein to ROS leads to spontaneous post-translational oxidation, which can render protein molecules dysfunctional [109]. In addition, mounting evidence has suggested that oxidative stress can contribute to the multi-factorial etiology of insulin resistance [49]. Oxidative stress occurs when the cellular production of reactive oxygen species (ROS) exceeds the level of anti-oxidant defenses within the cells. A number of studies have demonstrated increased oxidative stress in insulin-resistance and diabetes, including both increased

ROS production and reduced antioxidant capacity [8;63;83;116;119], purportedly related to the excessive metabolism of glucose and free fatty acids present in this physiologic state. For example, plasma levels of hydroperoxides (ROS) are higher in subjects with type 2 diabetes compared to nondiabetic subjects, and these levels are inversely correlated with the degree of metabolic control [81]. In addition, increased serum levels of several oxidation end-products, and particularly lipid peroxidation moieties, have been described in diabetic patients, as well as in several experimental animal models of diabetes [6;118]. Obesity alone is also associated with increased oxidative stress. In obese, non-diabetic subjects, lipid peroxidation (a marker of oxidant stress) directly correlates with waist circumference and body mass index (BMI), and inversely correlates with adiponectin, an insulin-sensitizing adipokine [37].

Oxidative stress also appears to modulate glucose transport through the insulin signaling pathway. Interestingly, low levels of exogenous H_2O_2 have been reported to mimic several physiologic responses of insulin, such as glucose transport [47;48;124] and glycogen synthesis [69]. Furthermore, stimulation of cells with insulin resulted in the generation of small amounts of H_2O_2 [75] via a NADPH oxidase-dependent system [74]. On the other hand, higher concentrations of H_2O_2 and longer exposure to oxidative stress serve to inhibit insulin action.

For example, prolonged exposure of 3T3-L1 adipocytes and L_6 myocytes to H_2O_2 significantly attenuates insulin-stimulated glucose transport and translocation of GLUT-4 to plasma membranes [95]. This effect was associated with decreased PI3-kinase and Akt activation and increased IRS-1 serine phosphorylation and degradation [88]. In vascular

smooth muscle cells, H_2O_2 decreased autophosphorylation of the insulin receptor and subsequent phosphorylation of Akt [39]. In addition, oxidative stress impairs the ability of insulin to stimulate the phosphorylation and de-activation of GSK-3 [96], which attenuates insulin signaling.

Oxidative stress is also implicated in diabetes-related mitochondrial dysfunction, both as a cause and a subsequent effect. As a consequence of normal mitochondrial function, the respiratory chain continually "leaks" electrons to oxygen to produce the free radical superoxide [43]. While relatively non-reactive itself, superoxide is the parent of other damaging ROS [9]. If ROS production exceeds the anti-oxidant protective mechanisms within the cell (the condition present in insulin-resistant and diabetic states), these reactive oxygen species lead to generalized oxidative damage to all mitochondrial components, including an increased mutation rate for mitochondrial DNA [59]. Mitochondrial DNA encodes 13 peptide components of oxidative phosphorylation complexes and the RNA machinery necessary for their translation [62]. Therefore dysfunction of mitochondrial DNA disrupts mitochondrial oxidative phosphorylation, and contributes to a number of human health problems, including insulin resistance [89] and diabetes [105]. Evidence suggests that hyperglycemia alone can cause mitochondrial dysfunction related to an increased exposure to oxidants [80], however, similar defects have also been demonstrated in the normoglycemic, insulin-resistant state. Insulin-resistant offspring of patients with type 2 diabetes have reduced rates of mitochondrial ATP production (30%), indicating a defect in oxidative phosphorylation [86]. Inefficient

mitochondrial ATP production in insulin-resistant tissues, like that shown in diabetic tissues, is likely related to increased ROS production and subsequent oxidative stress.

Taken together, these data suggest that obesity, insulin resistance, and diabetes are all states associated with high levels of ROS, and that this excess oxidant stress contributes to the dysregulation of glucose transport by attenuating normal insulin signaling (fig. 2).

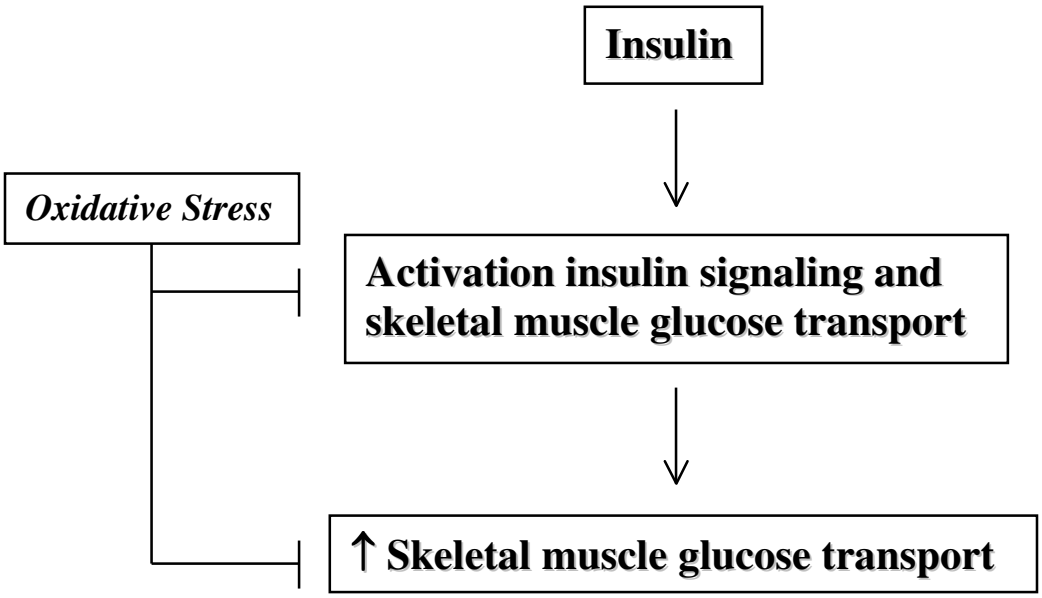


FIGURE 2

Figure 2. Potential inhibitory effects of oxidative stress on activation of insulin signaling and action of skeletal muscle glucose transport.

Moreover, it is possible that the condition of oxidative stress may contribute to the over-activity of GSK-3, and that the presence of both conditions together might cause greater insulin resistance than either alone. Consequently, selective inhibition of GSK-3 under the conditions of oxidative stress may mitigate the related defects in insulin action (fig. 3).

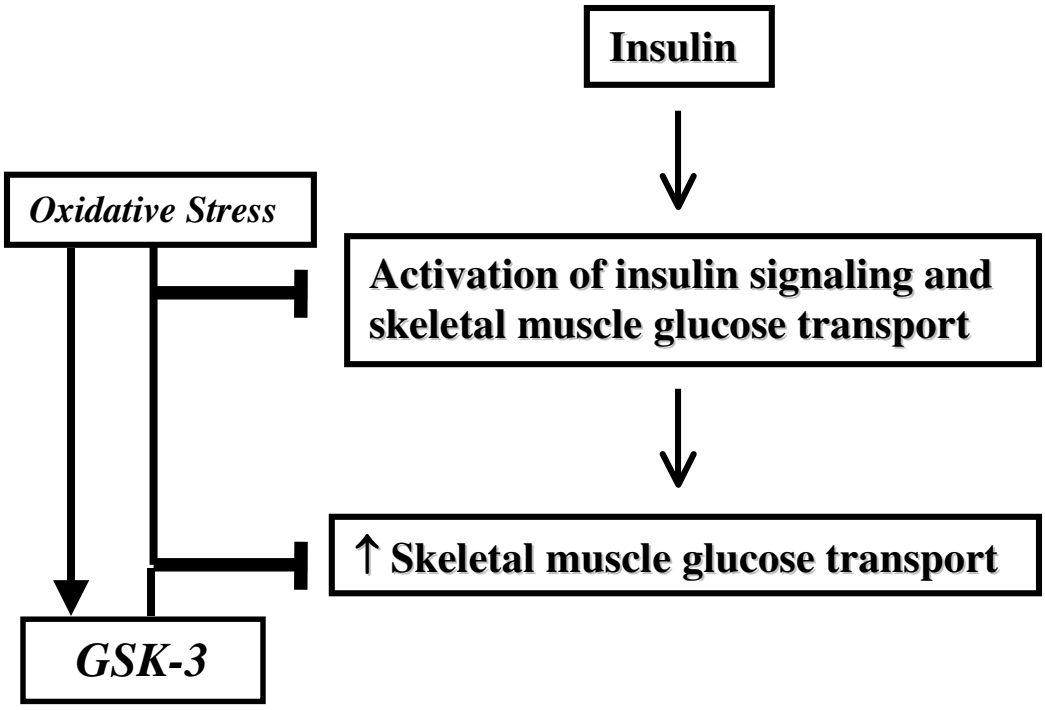


FIGURE 3

Figure 3. Potential additive inhibitory effects of oxidative stress and GSK-3 activity on activation of insulin signaling and action of skeletal muscle glucose transport.

Selective GSK-3 Inhibitors And Treatment of Glucose Dysregulation.

A substantial amount of effort has been devoted in the last decade to the development of compounds that can selectively target GSK-3 in the treatment of diabetes

(see recent reviews in refs. [30;110]. This strategy of targeting GSK-3 in this context is based on observations that overactivity and inadequate inhibitory control of GSK-3 has been linked to impaired insulin action [31;78]. The role of GSK-3 in metabolic regulation was initially investigated using lithium, until more selective GSK-3 inhibitors came available in the last five years.

Initial Investigations using Lithium. Lithium ions are a non-competitive and relatively non-selective inhibitor of GSK-3 with a K_i in the millimolar range [26;58;66;101]. In addition to GSK-3, lithium in this concentration range can also significantly inhibit inositol monophosphatase and adenylate cyclase [66], and potentially modulate a number of signaling pathways. Several investigations have addressed the potential beneficial effects of lithium on insulin-dependent glucose metabolism [2;94;98]. Direct incubation with lithium in the millimolar range can enhance both insulin-independent and insulin-dependent glucose transport in isolated skeletal muscle from insulin-sensitive rats [53;104] and type 2 diabetic rats [53]. Another ionic compound, zinc, has been shown to non-competitively inhibit GSK-3ß and induce an increase in glucose transport activity in mouse adipocytes [60]. However, because of the non-selective nature of lithium and zinc, the metabolic actions of these ions cannot be ascribed solely to inhibition of GSK-3. For this reason, several classes of highly selective GSK-3 inhibitors have been developed.

Selective GSK-3 Inhibitors: Effects on Whole-Body Glucose Disposal and Skeletal Muscle Glucose Transport.

A class of novel and selective organic inhibitors of GSK-3 has been developed by Chiron Corporation [91]. These Chiron GSK-3 inhibitors are substituted aminopyrimidine molecules and possess K_is of less than 10 nM for inhibition of either GSK-3 □ or GSK-3ß [91]. These compounds inhibit GSK-3 in an ATP-competitive manner [110], and are at least 500-fold more selective for GSK-3 than a panel of 25 metabolically-relevant kinases [91] The compounds CT98014, CT98030, and CT99021 activate glycogen synthase in the nanomolar range in cell lines [91], in isolated rodent skeletal muscle [53;91], and in cultured human myocytes [79]. The effects of these compounds on glycogen synthase activity are additive to that of insulin in insulin-sensitive skeletal muscle [53;91].

The metabolic actions of the substituted aminopyrimidine GSK-3 inhibitors have been extensively investigated in both in vitro and in vivo studies using animal models of insulin resistance and type 2 diabetes. In isolated skeletal muscles from the type 2 diabetic Zucker Diabetic Fatty (ZDF) rat [53;91], insulin action on glucose transport activity can be acutely potentiated with selective GSK-3 inhibitors. The potential mechanisms of action of the GSK-3 inhibitors in these isolated muscle experiments have been identified. In skeletal muscle from the ZDF rats, the enhanced insulin-stimulated glucose transport activity elicited by the GSK-3 inhibitor is associated with a greater amount of plasma membrane GLUT-4 protein, presumably due to a greater GLUT-4 translocation [53]. The effects of selective GSK-3 inhibition to potentiate insulin action on glycogen synthase activity and glucose transport have also been demonstrated in cultured muscle cells of type 2 diabetic human subjects [79]. Longer-term exposure of

these cultured human muscle cells to the GSK-3 inhibitors is associated with upregulation of IRS-1 and downregulation of GSK-3 protein expression [79].

When administered acutely to a variety of insulin-resistant rodent models, selective GSK-3 inhibitors cause a significant lowering of blood glucose, dramatically improve whole-body glucose tolerance and insulin sensitivity, and enhance insulin-stimulated skeletal muscle glucose transport activity in a variety of mouse and rat models of obesity-associated insulin resistance [53;91;110]. In addition, acute oral treatment with CT20026 improved oral glucose tolerance in mildly diabetic obese rhesus monkeys [110].

Investigations incorporating chronic administration of these GSK-3 inhibitors to animal models of insulin resistance and type 2 diabetes are scarce. However, one study demonstrated that treatment of type 2 diabetic ZDF rats with CT20026 for one month leads to a significant decrease in glycosylated hemoglobin (HBA1c), indicating an improvement of long-term glucose control in these hyperglycemic animals [110], therefore, further studies related to the effects of chronic GSK-3 inhibition are warranted

. Selective GSK-3 Inhibition and Hepatic Glucose Production.

Selective GSK-3 inhibition also appears to have important effects on liver glucose metabolism. Acute treatment of type 2 diabetic ZDF rats with a selective GSK-3 inhibitor significantly enhances glycogen synthase activity and glycogen synthesis in the liver [18]. This is consistent with the results of Lochhead et al. [70] who showed that a selective reduction of GSK-3 activity in rat hepatoma cells by treatment with maleimide-based GSK-3 inhibitors is associated with a reduction in the expression of the

gluconeogenic enzymes PEPCK and G6Pase [22]. Therefore, an enhancement of hepatic glycogen synthesis and a reduction in hepatic glucose output likely also contribute to the glucose-lowering effect of GSK-3 inhibitors in rodent models of type 2 diabetes.

Specific aims and hypotheses of this study

Despite the many facets of insulin resistance and oxidative stress that have been studied to date, there are still considerable gaps in our knowledge regarding these issues. For example, the role of GSK-3 in attenuating insulin signaling and insulin-regulated glucose metabolism is not clear, especially in the pre-diabetic, normoglycemic state. Moreover, the vast majority of previous experiments using selective GSK-3 inhibitors have used acute, rather than chronic, treatments. The beneficial effects of acute treatment with these agents on glucose transport and metabolism warrant further investigation using longer treatment periods. In addition, oxidative stress is associated with over-activity of GSK-3, but the potential relationship between these two pathological conditions has yet to be investigated.

Therefore, the specific aims and the associated hypotheses that will be tested are the following:

1. To determine the effects of acute, *in vitro,* selective GSK-3 inhibition in isolated skeletal muscle from insulin-resistant, pre-diabetic obese Zucker rats on basal and insulin-stimulated glycogen synthase activity and glucose transport, as well as on the functionality of critical proteins in the insulin signaling pathway.

Hypotheses:

- *Acute selective GSK-3 inhibition will increase basal and insulin-stimulated glycogen synthase activity and insulin-stimulated glucose transport in skeletal muscle.*
- *Acute selective GSK-3 inhibition will improve post-receptor insulin signaling, namely the functionality of IRS-1, PI3-kinase and Akt, and will decrease the activity of GSK-3 in skeletal muscle.*

2. To evaluate the effects of chronic, *in vivo* selective GSK-3 inhibition on whole-body insulin sensitivity, glucose tolerance, plasma free fatty acid concentration, and glycogen levels in liver and skeletal muscle.

   Hypothesis:

   - *Chronic, selective GSK-3 inhibition will improve whole-body insulin sensitivity and glucose tolerance, lower plasma free fatty acid concentrations, and increase glycogen levels in liver and skeletal muscle.*

3. To assess the effects of this chronic selective GSK3 inhibition in obese Zucker rats on basal and insulin-stimulated glucose uptake into skeletal muscle, and on protein expression of key insulin signaling elements in liver and skeletal muscle, and on the functionality of key insulin signaling elements in skeletal muscle.

   Hypothesis:

- *Chronic, selective GSK-3 inhibition in obese Zucker rats will increase insulin-stimulated glucose uptake into skeletal muscle, will up-regulate GLUT-4 and IRS-1 protein expression, and will improve post-receptor insulin signaling, namely the functionality of IRS-1, Akt, and PI3-kinase, and will decrease the activity of GSK-3 in skeletal muscle.*

4. To evaluate the role of GSK-3 in the insulin-resistant state of skeletal muscle induced by oxidative stress, including basal and insulin-stimulated glucose uptake, glycogen synthase activity and glycogen synthesis, and critical elements of the insulin signaling cascade.

   Hypotheses:

   - *In the presence of oxidative stress, selective GSK-3 inhibition will increase insulin-stimulated glucose uptake, glycogen synthase activity and glycogen synthesis in skeletal muscle.*

   - *In the presence of oxidative stress, selective GSK-3 inhibition will improve post-receptor insulin signaling, namely the functionality of IRS-1, PI3-kinase and Akt, and will decrease the activity of GSK-3 in skeletal muscle.*

CHAPTER 2

ACUTE SELECTIVE GLYCOGEN SYNTHASE KINASE-3 INHIBITION ENHANCES INSULIN SIGNALING IN PRE-DIABETIC INSULIN-RESISTANT RAT SKELETAL MUSCLE

Abstract

Glycogen synthase kinase-3 (GSK-3) has been implicated in the multifactorial etiology of skeletal muscle insulin resistance in animal models and in human type 2 diabetic subjects. However, the utility of selective GSK-3 inhibition in ameliorating this insulin resistance, and the molecular mechanisms involved, are not yet fully understood. Therefore, we determined if selective GSK-3 inhibition in vitro leads to an improvement in insulin action on glucose transport activity in isolated skeletal muscle of insulin-resistant, pre-diabetic obese Zucker (fa/fa) rats, and if these effects of GSK-3 inhibition are associated with enhanced insulin signaling. Type I soleus and type IIb epitrochlearis muscles from female obese Zucker rats were incubated in the absence or presence of a selective, small organic GSK-3 inhibitor (1 µM CT118637, K_i<10 nM for GSK-3 α and GSK-3 ß). Maximal insulin stimulation (5 mU/ml) of glucose transport activity, glycogen synthase activity, and select insulin signaling factors (tyrosine phosphorylation of insulin receptor (IR) and IRS-1, IRS-1 associated with p85 subunit of phosphatidylinositol-3-kinase, and serine phosphorylation of Akt and GSK-3) were assessed. GSK-3 inhibition enhanced (P <0.05) basal glycogen synthase activity and insulin-stimulated glucose transport in obese epitrochlearis (81% and 24%) and soleus (108% and 20%) muscles. GSK-3 inhibition did not modify insulin-stimulated tyrosine phosphorylation of IR ß-subunit in either muscle type. However, in obese soleus, GSK-3 inhibition enhanced (all P<0.05) insulin-stimulated IRS-1 tyrosine phosphorylation (45%), IRS-1 associated p85 (72%), Akt1/2 serine phosphorylation (30%), and GSK-3 ß serine phosphorylation (39%). Substantially smaller GSK-3 inhibitor-mediated enhancements of insulin action on these

insulin signaling factors were observed in obese epitrochlearis. These results indicate that selective GSK3 inhibition enhances insulin action in insulin-resistant skeletal muscle of the pre-diabetic obese Zucker rat, at least in part by relieving the deleterious effects of GSK3 action on post-insulin receptor insulin signaling. These effects of GSK3 inhibition on insulin action are greater in type I muscle than in type IIb muscle from these insulin-resistant animals.

Introduction.

Insulin resistance of skeletal muscle glucose disposal, resulting from defective myocellular insulin signaling, is the earliest and most prominent feature of the pre-diabetic state, a condition in which individuals are at great risk for the conversion to overt type 2 diabetes [34;88]. Type 2 diabetes is a world-wide epidemic, predicted to affect over 300 million people by the year 2025 [61]. Pre-diabetes, thought to affect ~40 million people in the United States, is identified by fasting plasma glucose levels between 100 and 125 mg/dl that do not reach the diagnostic criteria (≥126 mg/dl) for diabetes [3]. The insulin resistance in the pre-diabetic and type 2 diabetic states is multifactorial in nature (reviewed in Zierath [124], and the identification of specific defects in the insulin signaling cascade, which regulates the glucose transport process, is critical for the design of effect interventions for enhancing insulin action in these states.

Glycogen synthase kinase-3 (GSK3), a serine/threonine kinase that consists of highly homologous α- and ß-isoforms [117], phosphorylates and thereby inactivates glycogen synthase, resulting in reduced glycogenesis [25;102]. Overactivity and inadequate inhibitory control of GSK3 has been linked to impaired insulin action [32;78] (reviewed in [30;78;110]). Indeed, overexpression of GSK3 ß in skeletal muscle of male mice is associated with a marked decrease in glucose tolerance [85]. GSK-3 activity is also increased in skeletal muscle and adipose tissues of obese rodents [15;32] and in skeletal muscle of obese humans with type 2 diabetes [32;78], and this elevated GSK3 activity is associated with decreased insulin sensitivity [32;78]. Using in vitro assays, GSK3 has been shown to phosphorylate insulin receptor substrate-1 (IRS-1) on serine

residues [31], which would impair insulin action on glucose transport, suggesting that GSK-3 elicits a negative effect on the insulin signaling pathway due to impaired IRS-1 tyrosine phosphorylation. In addition to its deleterious effects on glucose transport and glycogen synthesis, GSK-3 can also increase gluconeogenic enzymes in the liver and contribute to the excess hepatic glucose production that is commonly present in type 2 diabetes [18;22;70].

Recently, a class of novel, selective organic inhibitors of GSK-3 has been developed [91] (reviewed in [20;110]. These substituted aminopyrimidine compounds are potent competitive inhibitors (acting at the ATP binding site) of human GSK-3 (K_i < 10 nM) with ≥ 500-fold selectivity against 20 other protein kinases [91], and have been shown to improve glycogen synthase (GS) activity, glucose tolerance, and glucose transport activity in rodent models of overt type 2 diabetes, such as the male Zucker Diabetic Fatty (ZDF) rat [53;91], and in cultured human muscle cells [79]. In vivo, acute administration of these compounds significantly reduces the elevated plasma glucose levels of the ZDF rat without elevating circulating insulin [53;91]. Interestingly, these selective GSK-3 inhibitors do not enhance insulin-stimulated glucose transport activity in skeletal muscle from animals with normal insulin sensitivity, such as the lean Zucker rat [53;91].

Although a beneficial effect of GSK-3 inhibition has been demonstrated in ameliorating skeletal muscle insulin resistance in animal models of type 2 diabetes, the utility of selective GSK-3 inhibition in mitigating skeletal muscle insulin resistance of glucose transport in the pre-diabetic state, and the molecular mechanisms involved, have

not yet been investigated. Therefore, the present investigation addressed the following specific aims: To determine the effects of acute, *in vitro,* selective GSK-3 inhibition in isolated skeletal muscle from insulin-resistant, pre-diabetic obese Zucker rats on basal and insulin-stimulated glycogen synthase activity and glucose transport, as well as on the functionality of critical proteins in the insulin signaling pathway, including the insulin receptor (IR), IRS-1, phosphatidylinositol-3-kinase (PI3-kinase), Akt, and GSK-3 .

Methods

GSK-3 inhibitor. The GSK-3 inhibitor CT118637 (kindly provided by Dr. Steve Harrison, Chiron Corporation, Emeryville, CA) is structurally very similar to and has identical pharmacokinetic properties to selective GSK-3 inhibitors used previously by our research group [53;91] (reviewed in [110]). It inhibits both GSK-3 α and GSK-3ß with K_i values less than 10 nM in an ATP-competitive manner (S. D. Harrison, personal communication). It was >95% pure by HPLC. In the in vitro incubations, the compounds 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 7AM 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 an 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 i.p. 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 30 min at 37°C in 3 ml 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 (radioimmunoassay grade, Sigma Chemical), 0.5% dimethyl sulfoxide, and the indicated additions of GSK3 inhibitor or insulin. Thereafter, the muscles were either blotted on filter paper and 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 glycogen synthase 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. [106], as modified by Henriksen et al. [55].

Assessment of glucose transport activity. After the initial 30-min incubation period, the muscles were rinsed for 10 minutes at 37°C in 3 ml of oxygenated KHB

containing 40 mM mannitol, 0.1% BSA, GSK3 inhibitor, and insulin, if present previously. Following 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/mmol; Sigma Chemical), 39 mM [U-^{14}C]mannitol (0.8 μCi/mmol; 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-[^{3}H]DG was determined as described previously [51]. This method for assessing glucose transport activity in isolated muscle has been validated [44].

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 Na pyrophosphate, 20 mM ß-glycerophosphate, 10 mM NaF, 2 mM Na_3VO_4, 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 PMSF). Homogenates were incubated on ice for 20 min and then centrifuged at 13,000 X g for 20 minutes 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-GSK3 α/ß ser$^{21/9}$ (Cell Signaling Technology) overnight. In our hands, ser^{21} phosphorylation of GSK3 α in muscle from the obese Zucker rat is very low (Dokken, B. B., J.A. Sloniger, and E.J. Henriksen, unpublished data), and all GSK3 data in this study are restricted to GSK3 ß ser^{9} phosphorylation. Subsequently, membranes were incubated with secondary goat anti-rabbit antibody conjugated with HRP (Chemicon, Temecula, CA). The proteins were visualized on Kodak X-Omat AR film (Kodak, Rochester, NY) using an enhanced chemiluminescence (ECL) 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.

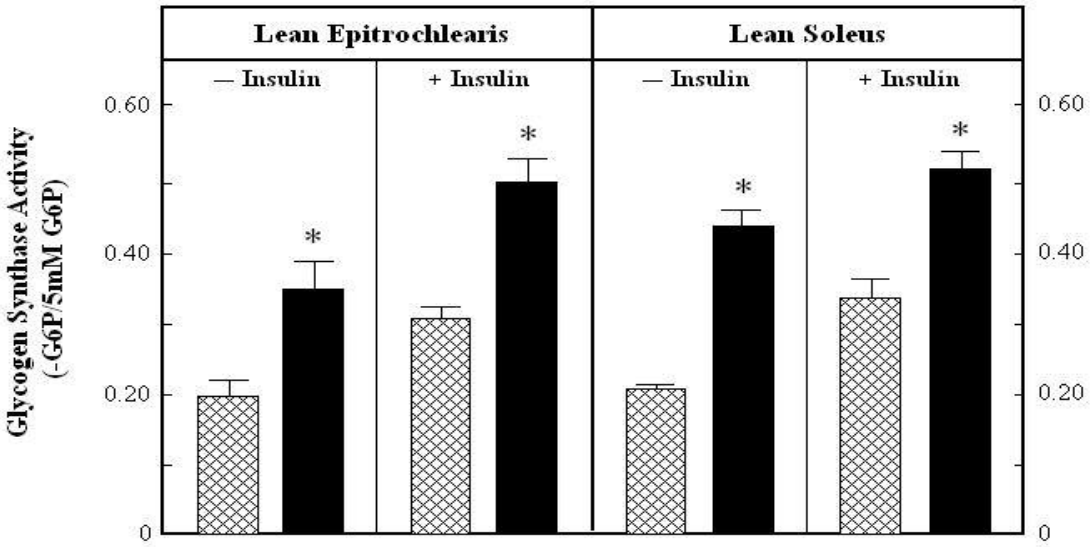
For measurement of tyrosine-phosphorylated IR-ß (IR/pY) and IRS-1 (IRS-1/pY) and for IRS-1 associated p85 (IRS-1/p85), immunoprecipitations and subsequent immunoblotting were performed. Muscles were homogenized in 1 ml of ice-cold lysis buffer and total protein concentration was determined as above. Samples were diluted to 2 mg/ml (IRS-1/pY and IR/pY) or 1 mg/ml (IRS-1/p85). For assessment of IR/pY, 0.5 ml of diluted homogenate was immunoprecipitated with 15 µl of recombinant agarose-conjugated anti-phosphotyrosine 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-PI3 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 minutes. Proteins were separated by SDS-PAGE on 7.5% polyacrylamide gels and transferred to nitrocellulose membranes. Equal loading of the gels for the protein of interest was assured. For detection of IR/pY and IRS-1/p85, membranes were incubated with the appropriate dilution of commercially available antibodies against insulin receptor ß-subunit (for IR/pY) 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.

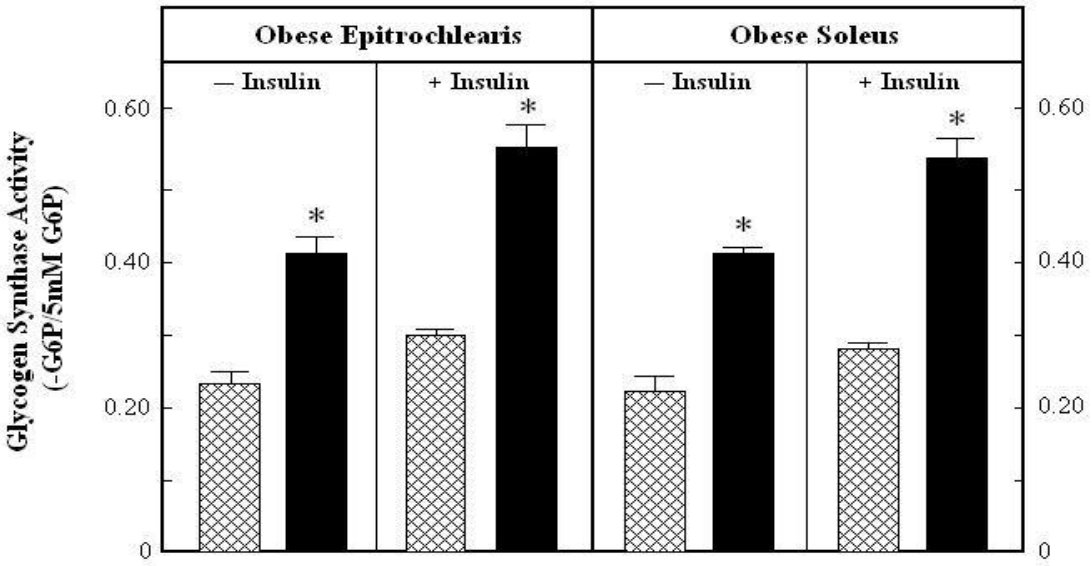
Statistical analysis. All values are expressed as means ± SE. All experiments were done with paired muscles from the same animal incubated with or without the GSK-3 inhibitor. Therefore, differences between the two groups due to the GSK-3 inhibitor alone (in the absence or presence of insulin) were analyzed using a paired Student's t-test. A level of $P<0.05$ was set for statistical significance.

Results

Effect of selective GSK-3 inhibition on glycogen synthase activity. To establish the effectiveness of CT118637 to inhibit GSK-3 in vitro, we assessed the modulation of the GS activity ratio in the absence or presence of the GSK-3 inhibitor in skeletal muscle from lean and obese Zucker rats (Fig. 4).



Panel A



Panel B

Fig. 4. Effects of GSK3 inhibitor CT118637 on basal or insulin-stimulated glycogen synthase activity ratio in skeletal muscle of lean (panel A) and obese (panel B) Zucker rats. Epitrochlearis and soleus strips were incubated without or with CT118637 (1 µM) in the absence or presence of insulin (5 mU/ml) for 30 min. The glycogen synthase activity ratio was calculated as the ratio of the activity in the absence of glucose-6-phosphate divided by the activity in the presence of 5 mM glucose-6-phosphate. Hatched bars are minus CT118637 and solid bars are plus CT118637. Values are means ± SE for 5 muscles per group. *P<0.05 vs. paired muscles in the absence of GSK3 inhibitor.

In the lean Zucker rats, 1 µM CT118637 enhanced (P<0.05) basal GS activity in epitrochlearis (88%) and soleus (106%) muscles (Fig 4A). 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 µM CT11837 enhanced (P<0.05) basal GS activity in both epitrochlearis (81%) and soleus (108%) muscles (Fig. 4B). 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 [53;91].

Effect of selective GSK3 inhibition on glucose transport activity.

In both epitrochlearis and soleus muscles from the lean Zucker rats, GSK-3 inhibition did not alter either basal or insulin-stimulated glucose transport activity (Fig. 5A).

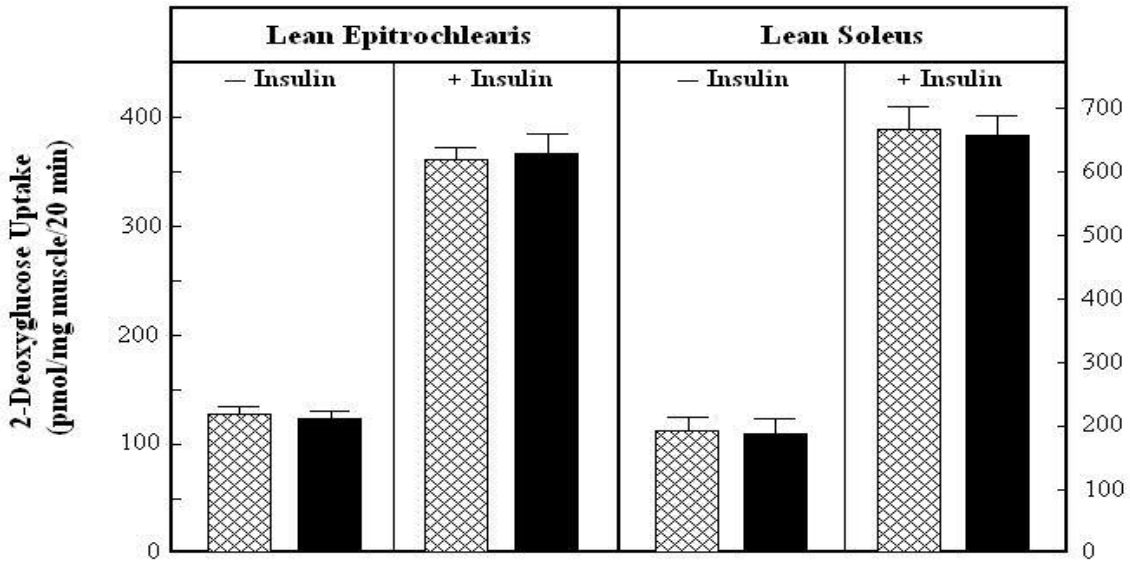


FIGURE 5A

Similarly, selective in vitro GSK-3 inhibition had no effect on basal glucose transport activity in epitrochlearis or soleus muscles from the obese Zucker rats (Fig. 5B).

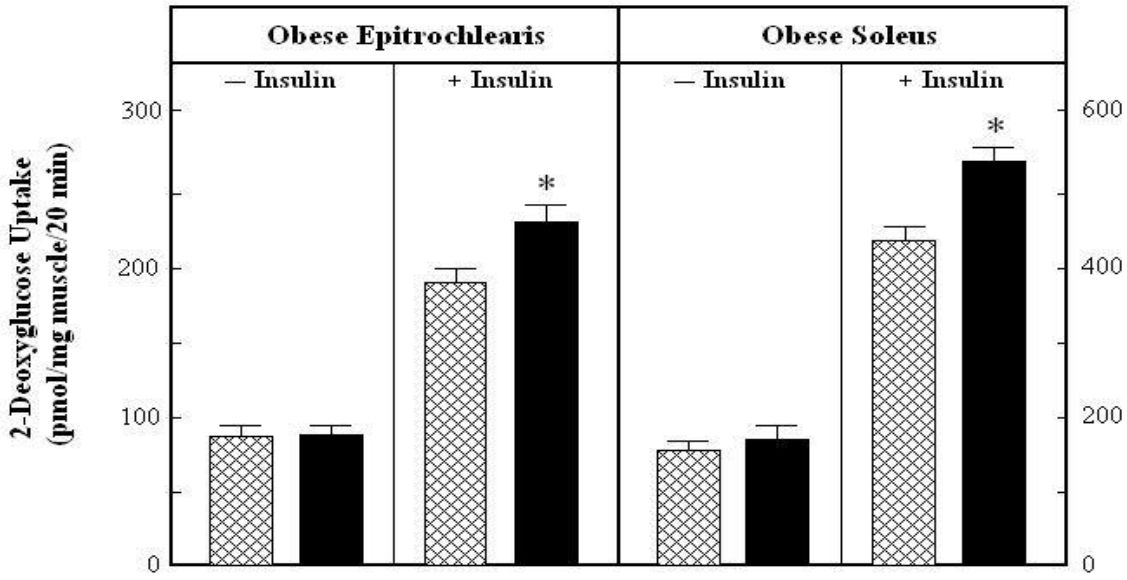


FIGURE 5B

Fig. 5. Effects of GSK3 inhibition on basal or insulin-stimulated glucose transport activity in skeletal muscle of lean (panel A) and obese (panel B) Zucker rats. Epitrochlearis and soleus strips were incubated without or with CT118637 (1 µM) in the absence or presence of insulin (5 mU/ml) for 30 min. 2-Deoxyglucose uptake was then performed as described in METHODS. Hatched bars are minus CT118637 and solid bars are plus CT118637. Values are means ± SE for 5 muscles per group. *P<0.05 vs. paired muscles in the absence of GSK3 inhibitor.

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 GSK-3 inhibition to enhance insulin-stimulated glucose transport activity was nearly twice as great in the obese type I soleus (87 ± 12 pmol/mg/20 min) as it was in the obese 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 [53;91], 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.

In order to identify a potential molecular mechanism responsible for the enhanced insulin-stimulated glucose transport activity in obese skeletal muscle due to GSK3 inhibition (Fig. 5B), the functionality of important elements of the insulin signaling cascade were assessed (Figs. 6-7). GSK3 inhibition did not affect either basal or insulin-stimulated tyrosine phosphorylation of the IR ß subunit in obese epitrochlearis and soleus muscles (Fig. 6).

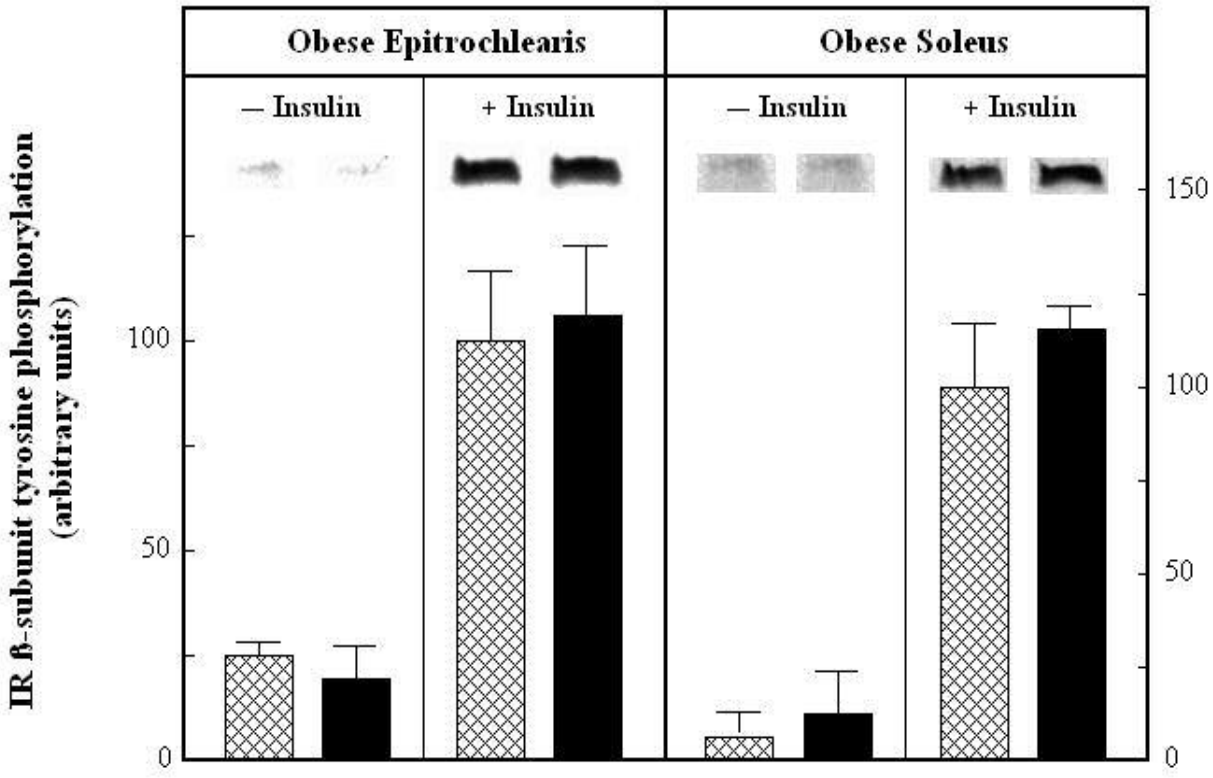


FIGURE 6

Fig. 6: Effect of GSK3 inhibition on basal or insulin-stimulated IR-ß tyrosine 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 mU/ml) for 30 min. Tyrosine phosphorylation of the ß-subunit of IR was then performed as described in METHODS. Representative bands are shown. Hatched bars are minus CT118637 and solid bars are plus CT118637. Values are means ± SE for 5-10 muscles per group. **P*<0.05 vs. paired muscles in the absence of GSK3 inhibitor.

In contrast, significant increases (*P*<0.05) in the level of IRS-1 tyrosine phosphorylation were observed in insulin-stimulated obese epitrochlearis (40%) and soleus (45%) muscles in the presence of the GSK-3 inhibitor (Fig. 7). Basal IRS-1 tyrosine phosphorylation was not affected by the GSK-3 inhibition.

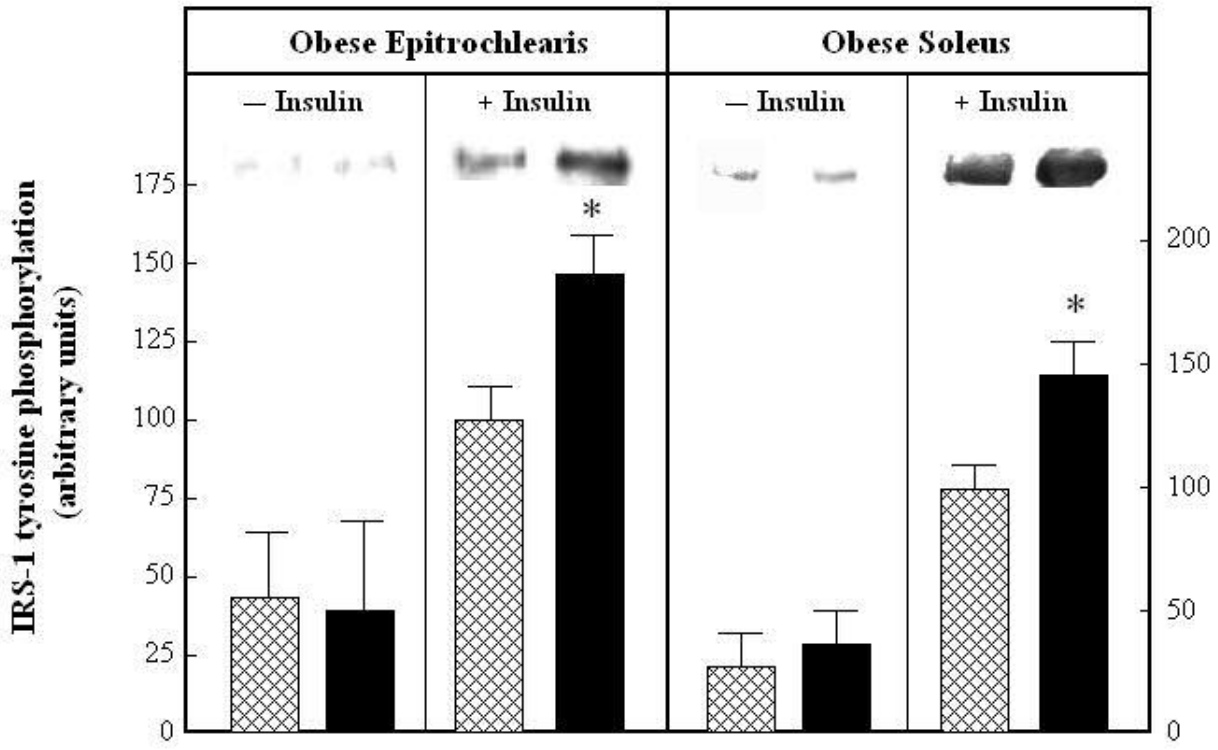


FIGURE 7

Fig. 7: Effect of GSK3 inhibition on basal or insulin-stimulated IRS-1 tyrosine 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 mU/ml) for 30 min. Tyrosine phosphorylation of IRS-1 was then performed as described in METHODS. Representative bands are shown. Hatched bars are minus CT118637 and solid bars are plus CT118637. Values are means ± SE for 5-10 muscles per group. *P<0.05 vs. paired muscles in the absence of GSK3 inhibitor.

The functionality of additional downstream elements of the insulin signaling cascade, including PI3-kinase, Akt, and GSK3 were assessed. No effects of GSK3 inhibition in the absence of insulin were observed for these factors (Fig. 8-10). However, in the obese soleus, GSK-3 inhibition in the presence of insulin induced significant

enhancements of IRS-1 associated with the p85 subunit of PI3-kinase (a surrogate measure of PI3-kinase activity) (72%), ser^{473} phosphorylation of Akt1/2 (30%), and ser^{9} phosphorylation of GSK-3ß (39%). GSK3 inhibition also increased the insulin stimulation of these factors in the obese epitrochlearis muscle, but these effects were less (44%, 21%, and 13%, respectively) than in the obese soleus muscle.

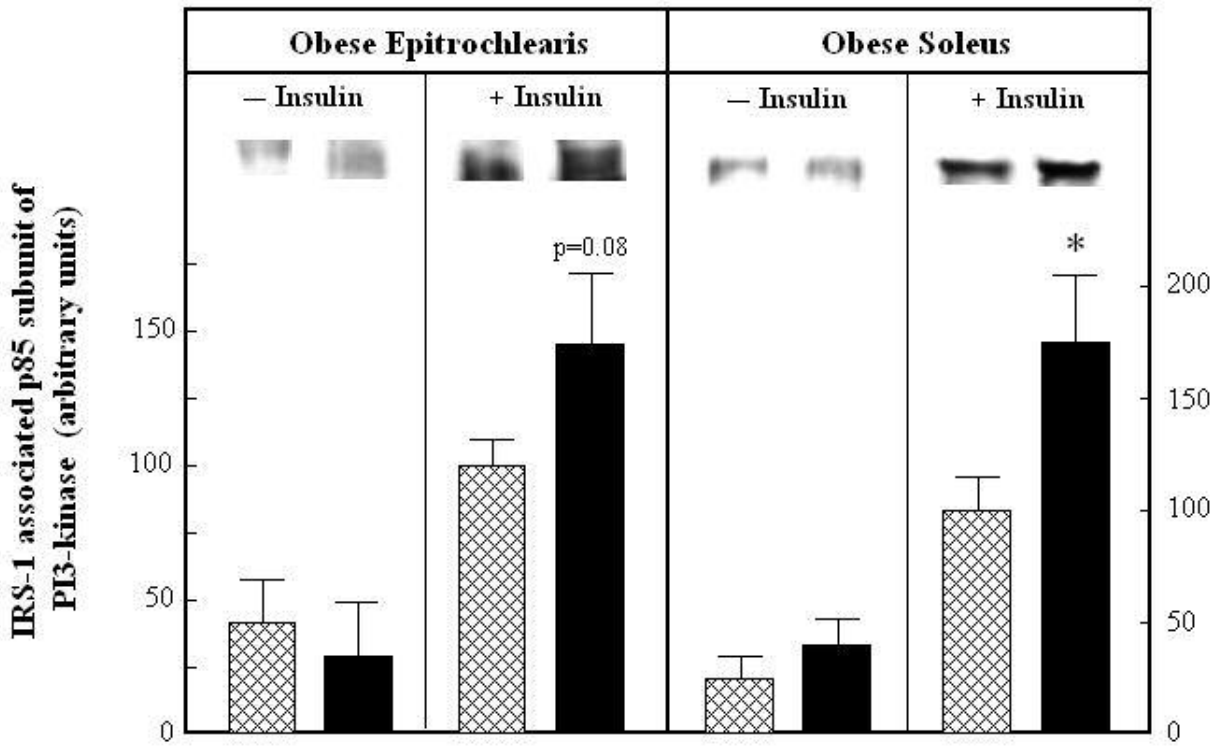


FIGURE 8

Fig. 8. Effect of GSK3 inhibition on basal or insulin-stimulated IRS-1 associated with p85 subunit of PI3-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 mU/ml) for 30 min. Assessment of IRS-1 associated with the p85 subunit of PI3-kinase was then performed as described in METHODS. Representative

blots are shown. Hatched bars are minus CT118637 and solid bars are plus CT118637. Values are means ± SE for 5 muscles per group. *P<0.05 vs. paired muscles in the absence of GSK3 inhibitor.

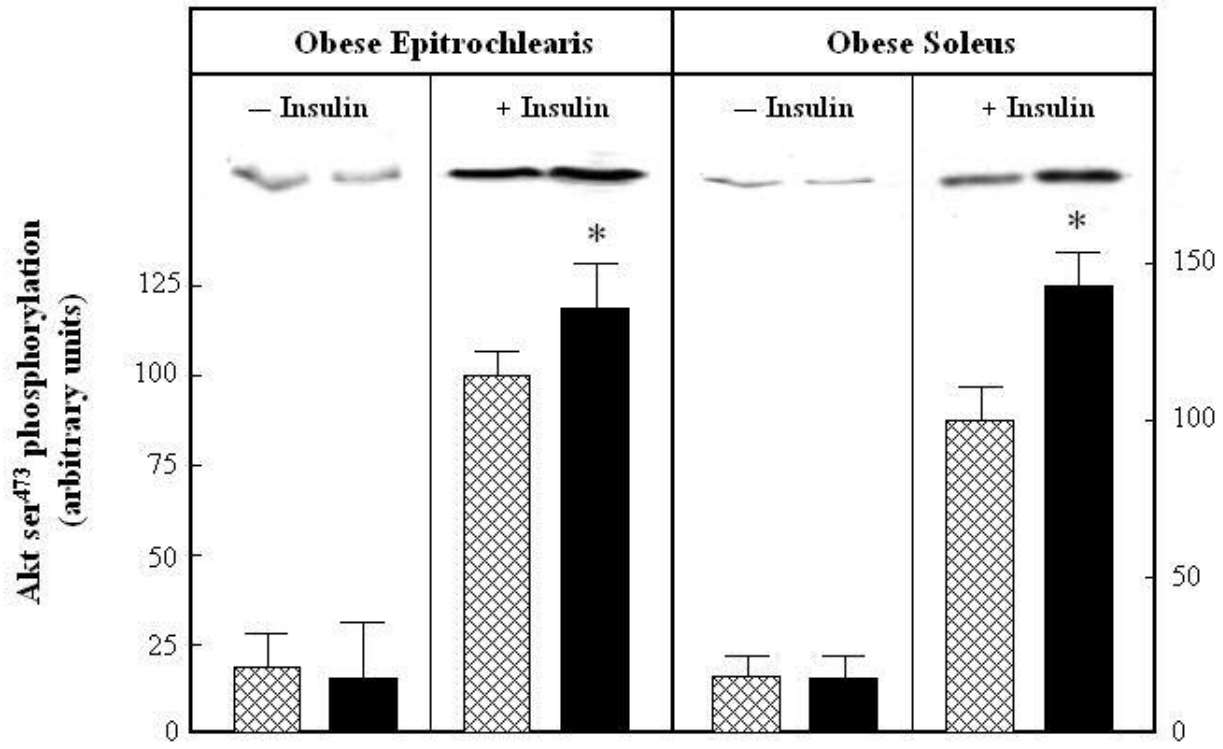


FIGURE 9

Fig 9. Effect of GSK3 inhibition on basal or insulin-stimulated Akt ser^{473} 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 mU/ml) for 30 min. Assessment of the phosphorylation of ser^{473} on Akt1/2 was then performed as described in METHODS. Representative bands are shown. Hatched bars are minus CT118637 and solid bars are plus CT118637. Values are means ± SE for 5-7 muscles per group. *P<0.05 vs. paired muscles in the absence of GSK3 inhibitor.

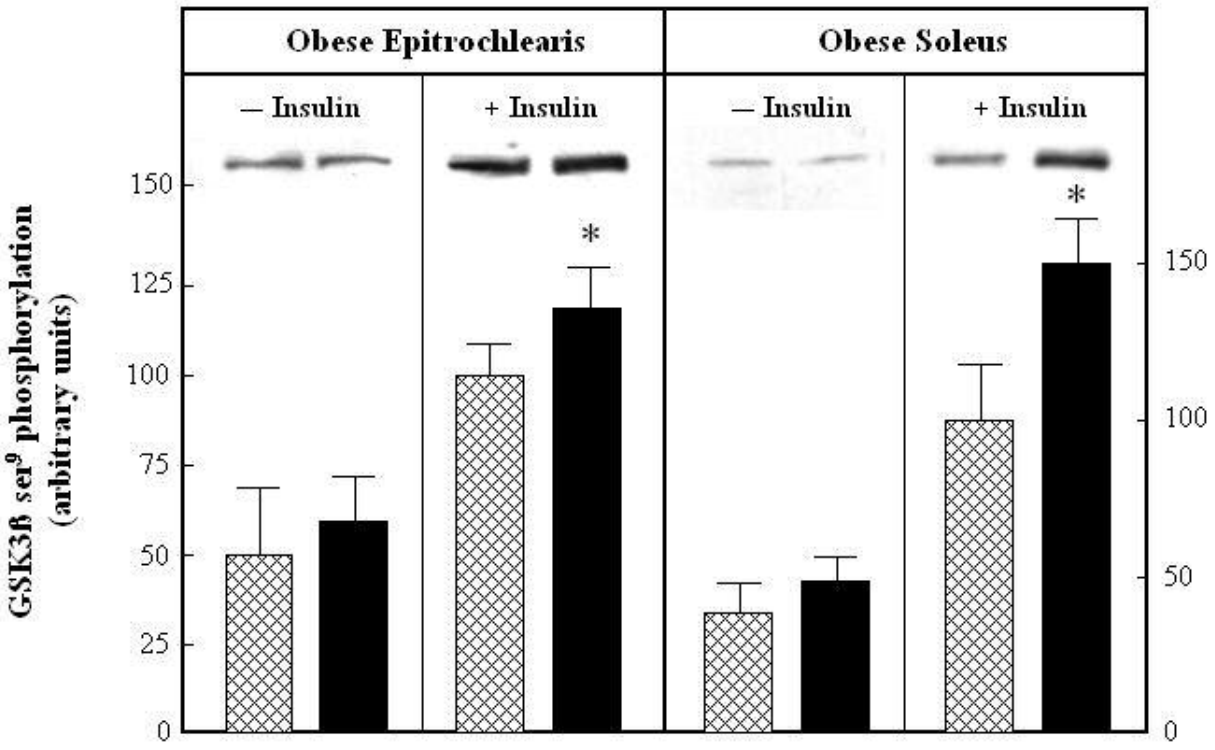


FIGURE 10

Fig 10. Effect of GSK3 inhibition on basal or insulin-stimulated GSK3ß ser^9 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 mU/ml) for 30 min. Assessment of the phosphorylation of ser^9 on GSK3ß was then performed as described in METHODS. Representative bands are shown. Hatched bars are minus CT118637 and solid bars are plus CT118637. Values are means ± SE for 5-10 muscles per group. *P<0.05 vs. paired muscles in the absence of GSK3 inhibitor.

Discussion

We have demonstrated in the present investigation that acute in vitro treatment of isolated skeletal muscle from insulin-resistant, pre-diabetic female obese Zucker rats with

the selective GSK3 inhibitor CT118637 can potentiate the ability of insulin to stimulate both glycogen synthase activity (Fig. 4) and glucose transport activity (Fig. 5). Importantly, although GSK-3 inhibition was manifest in skeletal muscle from both lean and obese animals, as evidenced by increases in the glycogen synthase activity ratio (Fig. 4), 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. 5). 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 [53;91] and not in insulin-sensitive tissues [53;70;91]. Moreover, these data support the hypothesis that elevated GSK3 activity, which exists in muscle from insulin-resistant, obese rodents [15;32] and humans with type 2 diabetes [78], 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. 7), a critical element of the insulin signaling cascade in muscle [124]. However, it is important to note that the enhancement of insulin action mediated by the GSK-3 inhibitor was clearly distal to the IR in obese skeletal muscle, as a similar GSK3 inhibitor-mediated upregulation of insulin action was not observed for tyrosine phosphorylation of the IR ß-subunit (Fig. 6). These results are in agreement with and complement the findings of Eldar-Finkleman and

colleagues [31], who demonstrated that overexpression of GSK-3 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 phoshosphorylation induced by the GSK-3 inhibitor appears to have important downstream effects in the insulin signaling cascade, as insulin action on IRS-1 associated with the p85 subunit of PI3-kinase (Fig. 8), ser^{473} phosphorylation on Akt (Fig. 9), and ser^{9} phosphorylation of GSK-3ß (Fig. 10) were similarly upregulated in skeletal muscle from the obese Zucker rat incubated with the selective GSK-3 inhibitor. Moreover, the enhancement of IRS-1 associated with p85 elicited by the GSK-3 inhibition is likely causally related to the increase in insulin-stimulated glucose transport activity, as the activation of PI3-kinase by tyrosine-phosphorylated IRS-1 is necessary for insulin-stimulated translocation of GLUT4 protein to the cell surface [31;124]. While GLUT4 protein translocation was not assessed in the present study, we have previously reported that in vitro GSK-3 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 [53].

These new data also support a mechanistic connection between the upregulation of Akt serine phosphorylation (Fig. 9) and GSK-3 ß serine phosphorylation (Fig. 10) and the potentiation of insulin stimulation of glycogen synthase activity (Fig. 4) due to the GSK-3 inhibition. The enhanced insulin-stimulated Akt activity (as reflected by the increased serine phosphorylation state) would catalyze greater GSK-3 phosphorylation,

and lead to less GSK3 activity, thereby further decreasing the ability of GSK3 to phosphorylate and inactivate glycogen synthase, a direct target of GSK-3 action [24]. Both the direct inhibitory effect of CT118637 on GSK3 and the reduced GSK3 activity due to covalent modifications of the enzyme resulting from enhanced Akt functionality can account for the increased insulin-stimulated activation of glycogen synthase in the presence of the GSK3 inhibitor CT118637. Collectively, these findings underscore the important physiological role of GSK3 in the regulation of glycogen synthase 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 compared to 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 [53]. One factor that may underlie this differential response to GSK3 inhibition is the fact that the protein expression and/or functionality of the insulin signaling factors of interest in the present study, including IRS-1 tyrosine phosphorylation, phosphotyrosine associated PI3-kinase activity, and Akt ser^{473} phosphorylation, are greater in type I muscle compared to type IIb muscle [100]. In addition, the protein expression of GLUT4 is likewise greater in type I soleus muscle than in type IIb epitrochlearis muscle [50]. 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, and 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 to type IIb muscle.

While 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 [53;87;91]. The disposal of an oral glucose load is significantly enhanced in ZDF rats treated with structurally and functionally similar GSK3 inhibitors (CT98023 and CT99021) [53;91]. These improvements in glucose tolerance were realized without a corresponding increase in the endogenous insulin level, indicating a reduction in insulin resistance [53;91]. 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 [91]. In addition, ip 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 [87]. Moreover, the recent finding by Pearce and colleagues [85] that selective overexpression of GSK3 ß 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.

In summary, selective in vitro GSK-3 inhibition with CT118637 increased insulin stimulation of glucose transport activity in insulin-resistant skeletal muscle of pre-diabetic, obese Zucker rats, but not in insulin-sensitive skeletal muscle of lean Zucker rats. The GSK-3- induced improvement of insulin action in the insulin-resistant, pre-diabetic skeletal muscle was associated with enhancement of IRS-1 tyrosine phosphorylation, IRS-1 associated with the p85 subunit of PI3-kinase, Akt ser^{473} phosphorylation, and GSK-3 ß ser^{9} phosphorylation, but not with IR tyrosine phosphorylation. Finally, the beneficial effects of GSK-3 inhibition on insulin action were generally greater in type I muscle (soleus) than in type IIb muscle (epitrochlearis) from these insulin-resistant, pre-diabetic obese animals. Collectively, these results provide support for selectively targeting GSK-3 in the treatment of skeletal muscle insulin resistance in the pre-diabetic state.

CHAPTER 3

CHRONIC SELECTIVE GLYCOGEN SYNTHASE KINASE-3 INHIBITION ENHANCES GLUCOSE DISPOSAL AND MUSCLE INSULIN ACTION IN PRE-DIABETIC OBESE ZUCKER RATS

Abstract

Increasing evidence supports a negative role of glycogen synthase kinase-3 (GSK-3) in the regulation of skeletal muscle glucose transport activity. We have shown previously that the acute treatment of insulin-resistant rodents with selective GSK-3 inhibitors improves whole-body insulin sensitivity and insulin action on skeletal muscle glucose transport. In the present investigation, we assessed the effects of chronic treatment of insulin-resistant, pre-diabetic obese Zucker (fa/fa) rats with a highly selective GSK-3 inhibitor (CT118637) on glucose tolerance, whole-body insulin sensitivity, plasma lipids, skeletal muscle insulin signaling, and in vitro skeletal muscle glucose transport activity. Obese Zucker rats were treated by gavage with either vehicle or CT118637 (30 mg/kg body weight) twice per day for 10 days, and studied 15-18 hours after the last treatment. Fasting plasma insulin and free fatty acid levels were reduced by 14% and 23% ($p<0.05$), respectively, in GSK-3 inhibitor-treated animals compared to vehicle-treated controls. The glucose response during an oral glucose tolerance test was reduced by 18% ($p<0.05$) and whole-body insulin sensitivity was increased by 28% ($p<0.05$) following chronic GSK-3 inhibition. In vivo IRS-1 tyrosine phosphorylation (50%) and IRS-1 associated phosphatidylinositol-3' kinase (79%) relative to fasting plasma insulin were significantly elevated ($p<0.05$) in plantaris muscles of GSK-3 inhibitor-treated animals. Whereas basal glucose transport in isolated soleus and epitrochlearis muscles was unaffected by chronic GSK-3 treatments, insulin stimulation of glucose transport above basal was significantly enhanced (32-60%, $p<0.05$). In summary, chronic treatment of insulin-resistant pre-diabetic obese Zucker rats with a

specific GSK-3 inhibitor enhances oral glucose tolerance and whole-body insulin sensitivity, and is associated with an amelioration of dyslipidemia and an improvement in IRS-1-dependent insulin signaling in skeletal muscle. These results provide further evidence that selective targeting of GSK-3 in muscle may be an effective intervention for the treatment of obesity-associated insulin resistance.

Introduction.

Insulin resistance of skeletal muscle glucose transport and metabolism is a hallmark of both the pre-diabetic state and overt type 2 diabetes [48;52;124]. The etiology of skeletal muscle insulin resistance is clearly multifactorial and can involve defective expression and functionality of multiple elements in the insulin signaling cascade that regulates the glucose transport process (see reviews in [48;52;124]). In both animal models of insulin resistance, such as the obese Zucker rat, and in humans with pre-diabetes or overt type 2 diabetes, there is diminished insulin-stimulated GLUT-4 protein translocation [33;124] and glucose transport activity [23;33;52;123;124] in skeletal muscle. This insulin resistance of muscle glucose transport is associated with well-defined defects in insulin signaling, including tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) and IRS-1-association with the p85 regulatory subunit of phosphatidylinositol-3' kinase (PI3-kinase) [4;12;40;56;97].

Glycogen synthase kinase-3 (GSK-3), a serine/threonine kinase consisting of highly homologous α and ß isoforms [117], can phosphorylate and inactivate glycogen synthase [84;92;122]. GSK-3 is constitutively active and is acutely deactivated by insulin signaling through the sequential activation of IRS-1, PI3-kinase, and ultimately via the action of Akt to phosphorylate specific serine residues on the enzyme [24]. GSK-3 can phosphorylate IRS-1 on serine and threonine residues, causing impairment of insulin signaling [31]. These observations support the hypothesis that GSK-3 can serve as a negative modulator of insulin action on glycogen synthase and, potentially, on glucose transport activity.

GSK-3 is overexpressed and is overactive in diabetic human and diabetic and pre-diabetic rodent skeletal muscle [29;78], and this inadequate inhibitory control of GSK-3 has been linked to impaired insulin action [32;78] (reviewed in [30;110]). Acute pharmacological inhibition of GSK-3 with substituted aminopyrimidine molecules increases insulin action on glycogen synthase and glucose transport in isolated muscle cells and tissues [29;53;79;91], likely due to enhanced IRS-1-dependent insulin signaling [29;53]. Acute administraton of these substituted aminopyrimidine GSK-3 inhibitors causes a significant lowering of blood glucose, dramatically improves whole-body glucose tolerance and insulin sensitivity, and enhances insulin-stimulated skeletal muscle glucose transport activity in a variety of mouse and rat models of obesity-associated insulin resistance [53;91;110]. In addition, acute oral treatment with this type of GSK-3 inhibitor improves oral glucose tolerance in mildly diabetic obese rhesus monkeys [110]. However, no other studies to date have directly evaluated the effect of chronic inhibition of GSK-3 activity on in vivo insulin action in mammalian skeletal muscle.

The present investigation was designed to test the overall hypothesis that chronic administration of a selective GSK-3 inhibitor would enhance insulin action in an animal model of obesity-associated insulin resistance. Therefore, the specific purposes of this study were 1) To evaluate the effects of chronic, *in vivo* selective GSK-3 inhibition on whole-body insulin sensitivity, glucose tolerance, plasma free fatty acid concentration, and glycogen levels in liver and skeletal muscle, and 2) To assess the effects of this chronic selective GSK3 inhibition in obese Zucker rats on basal and insulin-stimulated glucose uptake into skeletal muscle, and on protein expression of key insulin signaling

elements in liver and skeletal muscle, and on the functionality of key insulin signaling elements in skeletal muscle.

Methods

Animals, treatments, and oral glucose tolerance tests. Female obese Zucker (fa/fa) were purchased from Harlan (Indianapolis, IN) at the age of 8-9 wk and treatments were commenced at 10 wk of age. All animals were housed in a temperature-controlled room (20-22°C) with a 12:12-h light-dark cycle (lights on from 7AM 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.

The GSK-3 inhibitor CT118637 (kindly provided by Dr. Steve Harrison, Chiron Corporation, Emeryville, CA) is structurally very similar to and has identical pharmacokinetic properties to selective GSK inhibitors used previously by our research group [29;53;91] (reviewed in [110]). It inhibits both GSK-3α and GSK-3ß in vitro with K_i values less than 10 nM in an ATP-competitive manner [29;110]. Obese Zucker rats were treated by gavage with either vehicle or CT118637 (30 mg/kg; dissolved in 100 mM Tris buffer (pH 7.4)) twice daily (8 am and 5 pm) for 10 days, and studied 15-18 hours after the final treatments. This dose of GSK-3 inhibitor is based on its effectiveness to induce a significant effect on glucose tolerance and insulin sensitivity when administered acutely (up to 20 hr) (see refs. [18;53;91;110]). The acute administration of this class of GSK-3 inhibitor results in elevated levels of the inhibitor in plasma and skeletal muscle

for at least 4 hr (data not shown). Additional information on the pharmacokinetic properties of this class of GSK-3 inhibitors can be found in refs. [18;110].

On the 8th day, animals were food restricted (4 g of chow given at 5:00 PM the previous evening) and at 8:00 AM underwent an oral glucose tolerance test (OGTT) using a 1g/kg body wt glucose feeding by gavage. Blood (0.25 ml) was collected from a small cut at the tip of the tail immediately before and at 15, 30, 60 and 120 min after the glucose feeding. Whole blood was mixed thoroughly with EDTA (18 mM final concentration) and centrifuged at 13,000 g to isolate the plasma. The plasma was stored at -80°C and subsequently assayed for glucose (Sigma Chemical, St. Louis, MO), insulin (Linco Research, St. Charles, MO), and free fatty acids (FFA; Wako Chemicals, Richmond, VA). Fasting whole-body insulin sensitivity was estimated using the homeostasis model assessment of insulin resistance (HOMA-IR) by using the formula: [fasting plasma glucose (mg/dl) X fasting plasma insulin (μU/ml] /405 [18;77;110]. Immediately after completion of the OGTT, all animals received a 2.5 ml subcutaneous injection of 0.9% sterile saline to compensate for plasma loss, and treatments continued for two more days.

In vitro treatments of skeletal muscle. On the 11th day, after an 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 i.p. injection of pentobarbital sodium (50 mg/kg), and intact epitrochlearis muscles and strips of soleus muscles (~25-30 mg) were prepared for in vitro incubation in the unmounted state. In addition, unincubated strips of soleus, intact plantaris muscle, and a lateral lobe of the liver were removed and

immediately frozen in liquid nitrogen and subsequently used for assessment off insulin signaling (see below). The plantaris muscle is representative of type II muscle, consisting primarily of both type IIa and IIb fibers [107], whereas the soleus muscle is made up primarily of type I fibers [107]. Because the plantaris muscle is unsuitable for in vitro incubations, the epitrochlearis muscle was used instead for the assessment of glucose transport activity, as it also consists or predominantly type II fibers [93]. Soleus strips and epitrochlearis muscles were incubated for 60 min at 37°C in 3 ml 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 (radioimmunoassay grade, Sigma Chemical), without or with 5 mU/ml insulin. The incubated muscles were rinsed for 10 min at 37°C in 3 ml of oxygenated KHB containing 40 mM mannitol, 0.1% BSA, and insulin, if previously present. 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/mmol; Sigma Chemical), 39 mM [U-^{14}C] mannitol (0.8 μCi/mmol; ICN Radiochemicals, Irvine, CA), 0.1% BSA, 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, and weighed. These muscles were dissolved in 0.5 ml of 0.5 N NaOH, 5 ml of scintillation cocktail were added, and the specific intracellular accumulation of 2-deoxy-[1,2-^{3}H]glucose was determined as described previously [51]. This method for assessing glucose transport activity in isolated muscle has been validated [44].

Measurement of tissue glycogen concentration and glycogen synthase activity. For determination of glycogen [46], muscle was dissolved by heating in 1 ml of 5 N KOH. Glycogen was purified by ethanol precipitation, and then hydrolyzed to glucose by heating for 3 h at 100°C in 2 N HCl. After cooling, the sample was neutralized to pH 6 to 8 with 4 N NaOH, 0.1 M triethanolamine HCl, and assayed spectrophotometrically for glucose [11]. Glycogen synthase activity in plantaris 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. [106].

Assessment of insulin signaling factor protein expression and functionality. Frozen tissues were homogenized in 8 vol of ice-cold lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 20 mM Na pyrophosphate, 20 mM ß-glycerophosphate, 10 mM NaF, 2 mM Na_3VO_4, 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 PMSF). Homogenates were incubated on ice for 20 min and then centrifuged at 13,000 X g for 20 minutes at 4°C. Total protein concentration was determined using the BCA method (Sigma Chemical). Insulin signaling proteins were separated by SDS-PAGE on 7.5% or 12% polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA) and transferred to nitrocellulose membranes. To determine protein expression of insulin signaling factors, the blots were incubated with the commercially available antibodies against insulin receptor ß-subunit (IR-ß), insulin receptor substrates 1 and 2 (IRS-1 and IRS-2), the p85 regulatory subunit of phosphatidylinositol-3' kinase (PI3-kinase), GSK-3α/ß (Upstate

Biotechnology, Lake Placid, NY), and Akt1/2 (Cell Signaling Technology, Beverly, MA). Blots from skeletal muscle were also incubated with antibody against the GLUT-4 glucose ttransporter isoform (Biogenesis, Brentwood, NH). Membranes were then incubated with secondary goat anti-rabbit antibody conjugated with horse-radish peroxidase (HRP) (Chemicon, Temecula, CA). The proteins were visualized on Kodak X-Omat AR film (Kodak, Rochester, NY) using an enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia, Piscataway, NJ). The band intensities on the autoradiographs were quantified on a scanning densitometer (Bio-Rad Model GS-800) using Quantity One software (Bio-Rad).

For analysis of IRS-1/pY, 0.5 ml of diluted homogenate was immunoprecipitated with 25 µl of agarose-conjugated anti-IRS-1 antibody (Upstate Biotechnology). For analysis of IRS-1/p85, 0.5 ml of diluted homogenate was immunoprecipitated with 25 µl of agarose-conjugated anti-p85 antibody (Upstate Biotechnology). After an incubation period of 4 hours for IRS-1/p85, and overnight incubation at 4°C for IRS-1/pY, samples were centrifuged and the supernatant was removed. The beads were washed three times with ice-cold PBS, mixed with SDS sample buffer, and boiled for 5 minutes. Proteins were separated by SDS-PAGE on 7.5% polyacrylamide gels (Bio-Rad Laboratories) and transferred to nitrocellulose membranes. For analysis of IRS-1/pY, the nitrocellulose membrane was incubated in anti-phosphotyrosine antibody (PY99, Santa Cruz Biotechnology, Santa Cruz, CA). Immunoblotting for detection of

IRS-1/p85 was completed as described above using an antibody against IRS-1 (Upstate Biotechnology) 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.

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-GSK-3α/ß $ser^{21/9}$ (Cell Signaling Technology) overnight. In our hands, although the GSK-3α can be detected, ser^{21} phosphorylation of GSK-3α in muscle from the obese Zucker rat is very low (Dokken, B. B., J.A. Sloniger, and E.J. Henriksen, unpublished data), and all GSK-3 data in this study are restricted to GSK-3ß ser^{9} phosphorylation. Subsequently, membranes were incubated with secondary goat anti-rabbit antibody conjugated with HRP (Chemicon, Temecula, CA). Protein bands of interest were exposed to film, visualized, and quantified as described above.

Statistical analysis. All values are expressed as means ± SE. Differences between two groups were determined using an unpaired Student's *t*- test. A level of $P < 0.05$ was set for statistical significance.

Results

Effect of chronic selective GSK-3 inhibition on whole-body glucose tolerance and insulin sensitivity. The final average body weight of the obese Zucker rats treated chronically with the selective GSK-3 inhibitor was not different from that of the vehicle-treated animals (269 ± 8 g in vehicle-treated animals vs. 280 ± 7 g in CT118637-treated animals; 5 animals/group). Whereas fasting plasma glucose was not affected by the chronic GSK-3 inhibitor treatment, fasting plasma insulin (-14%) and free fatty acid (-23%) levels were significantly (P<0.05) lower in the obese animals treated with CT118637 compared to the vehicle-treated obese rats (Table 1). The HOMA-IR value, inversely related to insulin sensitivity in the fasting state [77], was significantly reduced in the GSK-3 inhibitor-treated group (Table 1), indicating that the chronic GSK-3 inhibition enhanced fasting whole-body insulin sensitivity.

Table 1. Effects of chronic GSK-3 inhibitor treatment on fasting plasma glucose, insulin, free fatty acids, and whole-body insulin sensitivity in obese Zucker rats.

Group	Plasma Glucose (mg/dl)	Plasma Insulin (µU/ml)	Plasma Free Fatty Acids (mM)	HOMA-IR (units)
Obese Vehicle-treated	**116 ± 7**	**134 ± 3**	**1.46 ± 0.08**	**38.3 ± 2.4**
Obese GSK-3 inhibitor -treated	**116 ± 3**	**115 ± 5 ***	**1.13 ± 0.10 ***	**32.0 ± 1.6***

Values are means ± SE for 5 animals per group. HOMA-IR units are mg/dl X µU/ml. * P <0.05 vs. obese vehicle-treated controls.

There was no long-lasting effect of the GSK-3 inhibition on fasting glycogen concentrations in liver (38 ± 3 vs. 39 ± 3) and plantaris muscle (15 ± 2 nmol/mg vs. 15 ± 3), and there was no effect of the GSK-3 inhibitor treatment on total activity of glycogen synthase in this skeletal muscle (1.2 ± 0.2 nmol/mg/min vs. 1.2 ± 0.2). Although the absolute glycogen synthase activity ratio in plantaris muscle (0.38 ± 0.04 vs. 0.38 ± 0.05) did not differ between the vehicle-treated group and the GSK-3 inhibitor-treated group, when expressed relative to the prevailing plasma insulin level, the glycogen synthase activity ratio was significantly greater ($P<0.05$) in the GSK3 inhibitor-treated group (3.3 ± 0.2 ml/μU X 10^{-3}) compared to the vehicle-treated control group (2.8 ± 0.2).

The glucose and insulin responses during the oral glucose tolerance test are shown in Fig. 11 (top panels). The glucose response to the oral glucose load was markedly reduced ($P<0.05$) in the chronic GSK-3 inhibitor-treated group at the 15-min (23%) and 30-min (15%) time points and remained reduced up to 120 min. The insulin response was likewise blunted in the chronic GSK-3 inhibitor-treated group, with a 25% reduction ($P<0.05$) at the 15-min time point. The total area under the glucose curve (AUC_g) was significantly reduced (18%, $P<0.05$) by the chronic GSK-3 inhibitor treatment (Fig. 11, bottom left panel). The total insulin area under the curve (AUC_i) was also slightly reduced in this group compared to the vehicle-treated control group (Fig. 11, bottom center panel), but this difference did not reach statistical significance.

Whole-body insulin sensitivity was also assessed from the OGTT data by determining the reciprocal of the glucose-insulin index, defined as the product of the AUC_g and the AUC_i and inversely related to whole-body insulin action [21]. This whole-

body insulin sensitivity index was enhanced by 28% ($P<0.05$) in the chronic GSK-3 inhibitor-treated group (Fig. 11, bottom right panel).

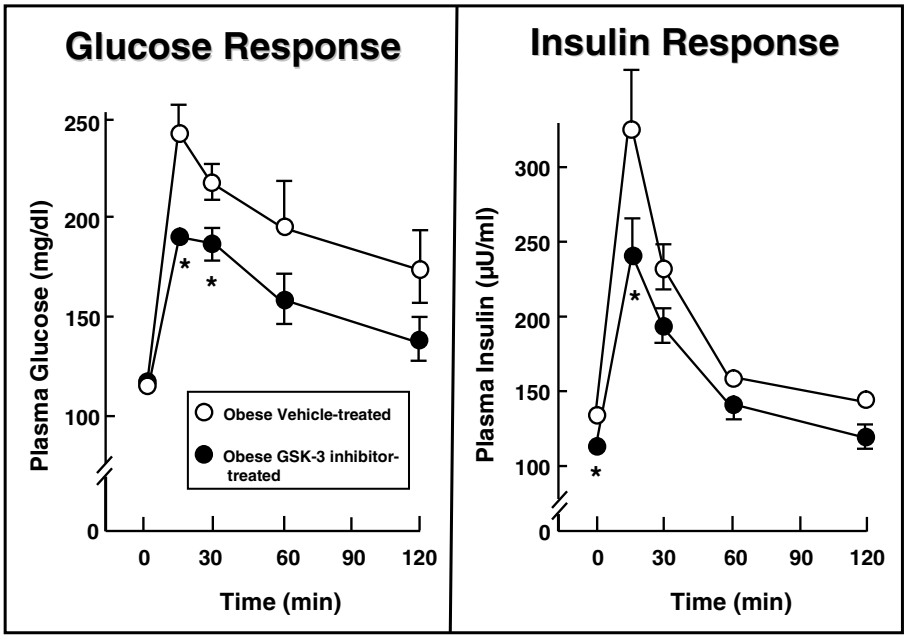


FIGURE 11 (TOP)

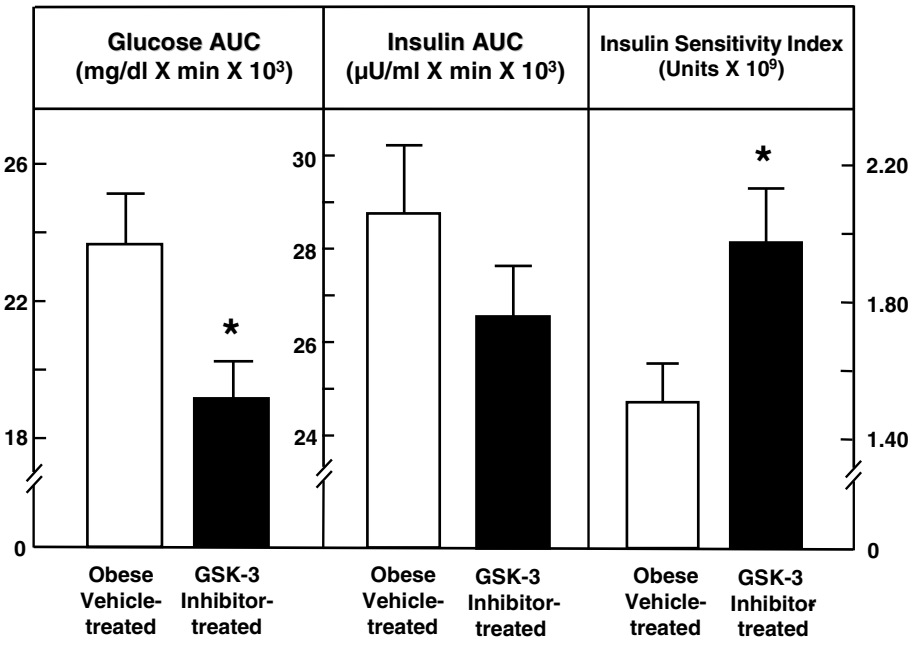


FIGURE 11 (BOTTOM)

Fig. 11. Top panels: Effects of chronic treatment of obese Zucker rats with GSK-3 inhibitor CT118637 on glucose and insulin responses during an oral glucose tolerance test. Bottom panels: Effects of chronic treatment of obese Zucker rats with GSK3 inhibitor CT118637 on glucose (AUC_g) and insulin (AUC_i) total areas under the curve and whole-body insulin sensitivity during an oral glucose tolerance test. Whole-body insulin sensitivity was calculated as the inverse of the product of (AUC_g) and (AUC_i). Error bars not shown in the top panels are smaller than the circles. Values are means ± SE for 5 animals per group. *P<0.05 vs. obese vehicle-treated group at same time point in the top panels.

Effect of chronic selective GSK-3 inhibition on in vivo muscle insulin signaling. The chronic treatment of the obese Zucker rats with the selective GSK-3 inhibitor did not alter the protein expression of insulin receptor ß-subunit, IRS-1, IRS-2, the p85 subunit of PI3-kinase, Akt, GSK-3, or the GLUT-4 glucose transporter in soleus and plantaris muscles (data not shown). In addition, protein expression of these various insulin signaling factors was not altered in liver of these GSK-3 inhibitor-treated animals (data not shown).

The in vivo functional states of IRS-1 and PI3-kinase were assessed in skeletal muscle of the vehicle-treated and chronic GSK-3 inhibitor-treated obese animals (Figs. 12 and 13).

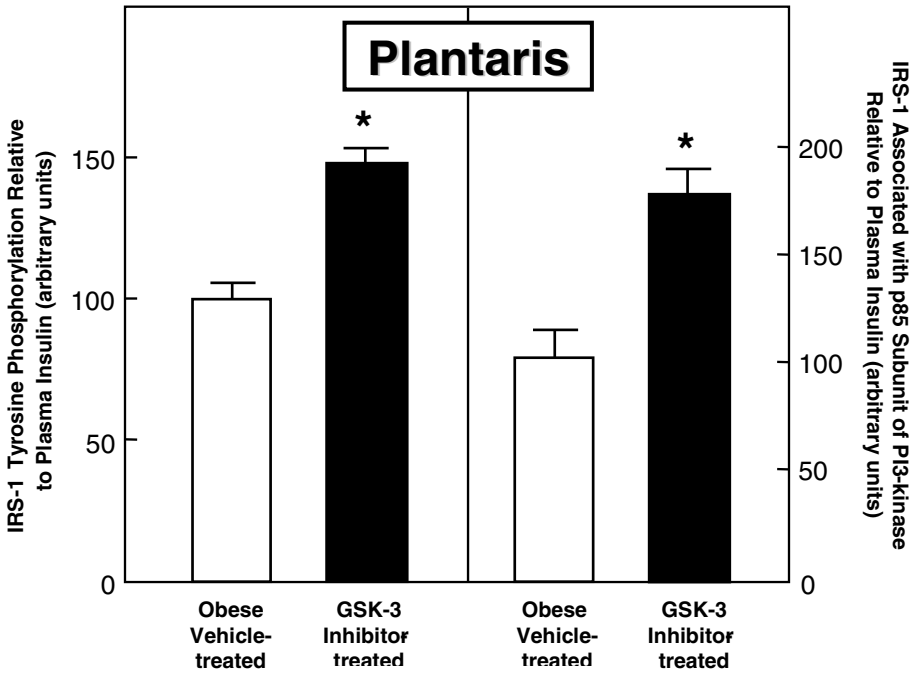


FIGURE 12

Fig. 12: Effects of chronic treatment of obese Zucker rats with GSK-3 inhibitor CT118637 on in vivo IRS-1 tyrosine phosphorylation and IRS-1 associated with the p85 subunit of PI3-kinase in plantaris muscle. Values are means ± SE for 4-5 muscles per group. *P<0.05 vs. the obese vehicle-treated group.

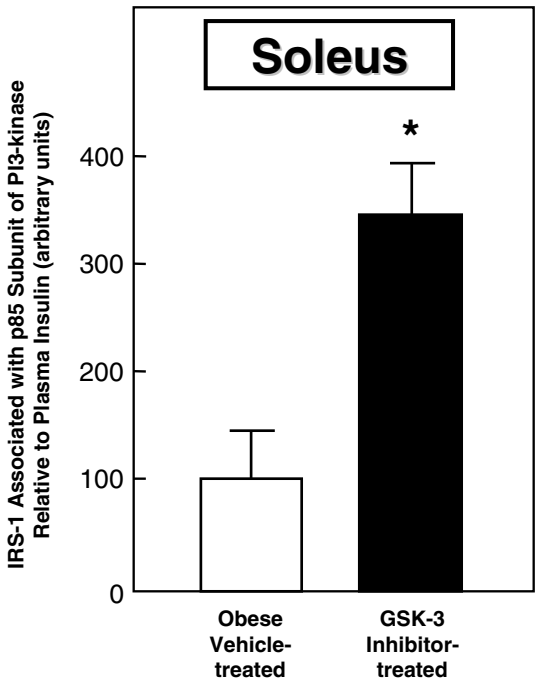


FIGURE 13

Fig. 13: Effects of chronic treatment of obese Zucker rats with GSK-3 inhibitor CT118637 on in vivo IRS-1 tyrosine phosphorylation and IRS-1 associated with the p85 subunit of PI3-kinase in soleus muscle. Values are means ± SE for 4-5 muscles per group. *P<0.05 vs. the obese vehicle-treated group.

In the plantaris, IRS-1 tyrosine phosphorylation (79%) and IRS-1 associated with p85 (50%), a surrogate measure of PI3-kinase activity, were both significantly (P<0.05) greater in the GSK-3 inhibitor-treated group when expressed either in absolute terms (data not shown) or relative to the fasting plasma insulin concentration (Fig 12). In the soleus, IRS-1 associated with the p85 subunit of PI3-kinase was significantly elevated 250%, P<0.05) when expressed relative to the prevailing in vivo plasma insulin level (Fig. 13). No differences in the phosphorylation states of Akt or GSK-3β between the vehicle-treated and GSK-3 inhibitor-treated groups were detected in these muscles, whether these variables were normalized to total protein or to the expression of the specific protein (data not shown).

Effect of chronic selective GSK-3 inhibition on in vitro muscle glucose transport activity. Like the plantaris, the epitrochlearis is composed of primarily type II fibers [57]. Basal and insulin-stimulated glucose transport activities were assessed in isolated type II epitrochlearis and type I soleus muscles from the vehicle-treated and chronic GSK-3 inhibitor-treated obese Zucker rats. As shown in Fig. 14, there was no difference between treatment groups for basal glucose transport activity in either muscle type. In contrast, the effect of a maximally-effective concentration of insulin to stimulate glucose transport activity above basal was significantly ($P<0.005$) enhanced in both soleus (32%, fig. 14A) and epitrochlearis (60%, Fig. 14B) muscles of the chronic GSK-3 inhibitor-treated animals.

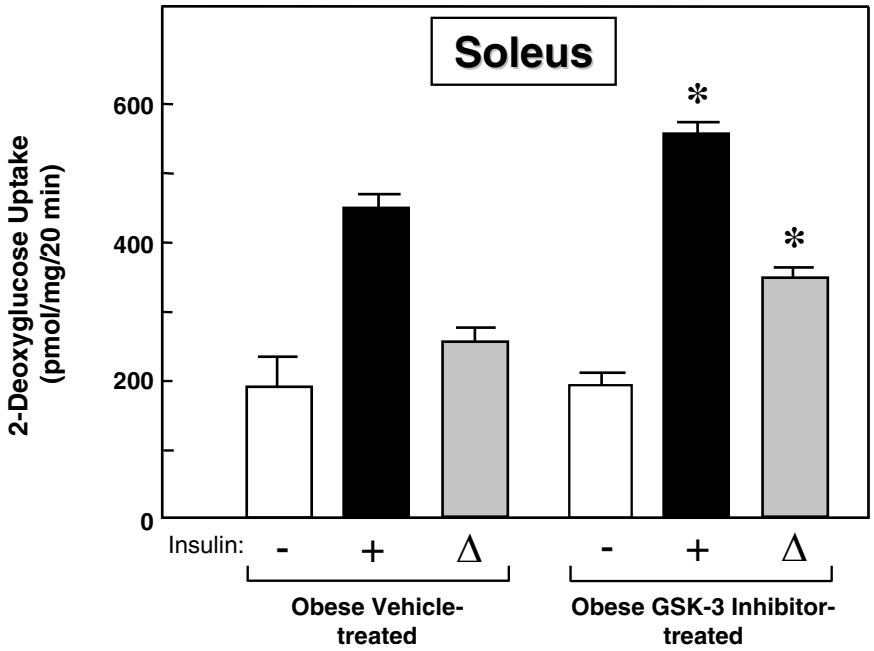


FIGURE 14A

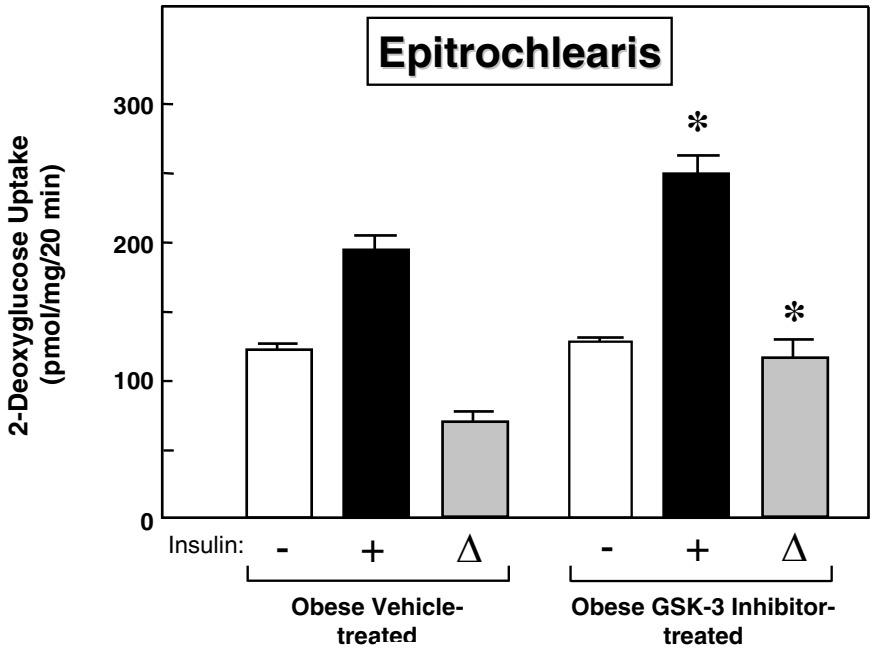


FIGURE 14B

Fig. 14. Effects of GSK-3 inhibition on basal or insulin-stimulated glucose transport activity in skeletal muscle of obese Zucker rats treated chronically with GSK-3 inhibitor CT118637. Epitrochlearis and soleus strips were incubated without (open bars) or with insulin (5 mU/ml) (black bars) for 60 min. 2-Deoxyglucose uptake was then performed as described in METHODS. Values are means ± SE for 4-5 muscles per group. *P<0.05 vs. the obese vehicle-treated group.

Discussion

In the present investigation, we have made the novel finding that chronic oral treatment of pre-diabetic, insulin-resistant obese Zucker rats with a selective, organic inhibitor of GSK-3 improves glucose tolerance and whole-body insulin sensitivity (Fig. 11 and Table 1). Previous investigations using the Zucker Diabetic Fatty (ZDF) rat, a related model of overt type 2 diabetes, had demonstrated that acute (4-hr) oral treatment with [18;53;91] or short-term (20-hr) infusion [18] of these substituted aminopyrimidine-

based GSK-3 inhibitors enhanced oral glucose tolerance and whole-body insulin sensitivity. The findings in the present investigation now indicate that longer-term beneficial effects of GSK-3 inhibition on glucoregulation can be realized in a rat model of severe insulin resistance, even 15-18 hours after the last oral administration of the GSK-3 inhibitor.

The enhancement of whole-body insulin sensitivity in the fasting state elicitied by the chronic GSK-3 inhibitor treatment in the obese Zucker rats (i.e., reduced HOMA-IR, Table 1) was associated with significant improvements in the functional status of key elements of the insulin signaling cascade in muscle, namely tyrosine phosphorylation of IRS-1 and IRS-1 association with the p85 subunit of PI3-kinase (Figs. 12 and 13). Moreover, the maximal capacity for insulin-stimulation of glucose transport activity was also upregulatd in type 1 soleus and type II epitrochlearis muscles of the chronic GSK-3 inhibitor-treated obese animals (Fig. 14). These alterations in IRS-1-dependent signaling and glucose transport activity are similar to those observed by our group following acute in vitro treatment of skeletal muscle from obese Zucker rats with this same GSK-3 inhibitor [29]. However, it is unlikely that these enhancements in insulin action on the skeletal muscle glucose transport system were induced by the last administration of GSK-3 inhibitor, as the acute effects of these substituted aminopyrimidine compounds are largely gone 4 hours after the final gavage treatment [53] at least in ZDF rats. Indeed, at the tie that muscle glucose transport activity was assessed, muscle and liver glycogen levels were the same as in the vehicle-treated obese animals.

The chronic oral treatments with the selective GSK-3 inhibitor caused a significant decrease in plasma FFA levels (Table 1). It is possible that this improvement in the lipemic state of the obese Zucker rats is mechanistically associated with the enhanced whole-body insulin sensitivity. It is known that FFAs or their derivatives can directly and indirectly inhibit the functionality of IRS-1-dependent insulin signaling in skeletal muscle [121]. Therefore, a decrease in the plasma levels of these inhibitory lipids following chronic GSK-3 inhibitor treatment could allow for greater signaling via IRS-1-dependent steps, such as PI3-kinase (Fig. 13), and a similar enhancement of muscle glucose disposal.

The chronic GSK-3 inhibition upregulated the functionality of IRS-1 and PI3-kinase in muscle in the absence of any significant alterations in the protein expression of a wide variety of insulin signaling factors and GLUT-4. These data are in contrast to the findings of Nikoulina et al. [79], who showed that long-term exposure of cultured human myocytes to a similar substituted aminopyrimidine-based GSK-3 inhibitor caused a downreguation of GSK-3 protein expression and an upregulation of IRS-1 protein expression. One important difference between these two studies is that the obese Zucker rats in the present study were exposed twice daily to a large dose of GSK-3 inhibitor, whereas the cultured myocytes were exposed continually to a constant concentration of the GSK-3 inhibitor. It is clear that intermittent exposure to the GSK-3 inhibitor in vivo is insufficient to elicit long-lived changes in protein expression of these insuli signaling factors and GLUT-4 in tissues of these pre-diabetic rats.

To our knowledge, this is the only investigation to date that has addressed the chronic effects of GSK-3 inhibition in a rat model of pre-diabetes. However, in a recent study, Kaidanovich-Beilinand and Eldar-Finkelman [64] investigated the consequences of the chronic intraperitoneal administration of a novel competitive peptide inhibitor of GSK-3 in ob/ob mice. In agreement with the present study, the chronic GSK-3 inhibition in the ob/ob mice caused a significant improvement of glucose tolerance. However, these investigators also reported increased hepatic IRS-2 protein expression and glycogen concentration, whereas in skeletal muscle GLUT-4 protein expression was enhanced and glycogen levels were slightly elevated [64], findings that were not corroborated in the present study using obese Zucker rats. There are clearly some different chronic adaptive responses to GSK-3 inhibition in rat and mouse models of glucose dysregulation.

Related to these investigations are the results from a recent investigation utilizing muscle-specific GSK-3 transgenic mice [79;85;85]. Selective overexpression of GSK-3β in skeletal muscle of male mice was associated with an increase in fat mass, a decrease in muscle IRS-1 protein expression, and decreased glycogen synthase activity and glycogen levels in muscle. These muscle-specific GSK -3-β transgenic mice were also characterized by marked glucose intolerance and hyperinsulinemia, consistent with reduced whole-body insulin sensitivity, and by elevated plasma FFA and triglycerides [79;85]. These findings are consistent with the interpretation that overactivity of GSK-3 specifically in skeletal muscle is associated with whole-body insulin resistance, hyperinsulinemia, and dyslipidemia, similar to the defects observed in the obese Zucker rat.

While the present investigation has focused primarily on the consequences of chronic GSK-3 inhibitor administration on the skeletal muscle glucose transport system, the potential contribution of altered hepatic glucose production to the improvement of glucoregulation following GSK-3 inhibition must also be discussed. Short-term infusion of these aminopyrimidine-based GSK-3 inhibitors in the type 2 diabetic ZDF rats significantly reduces hepatic glucose production [18]. Importantly, chronic administration of a peptide-based competitive inhibitor of GSK-3 in insulin-resistant ob/ob mice reduced hepatic mRNA levels of phosphoenolpyruvate carboxykinase (PEPCK), and important enzyme involved in hepatic gluconeogenesis, possibly due to a diminution of cAMP-responsive element binding (CREB) protein, a transcription factor critical in the regulation of PEPCK gene expression [64]. This finding is consistent with cell-based studies demonstrating downregulation of PEPCK gene expression following in vitro exposure to maleimide-based GSK-3 inhibitors [22;70;70]. These data indicate that in insulin-resistant states an additional important action of GSK-3 inhibitors is a reduction of hepatic glucose production, likely mediated by downregulation of genes associated with gluconeogenesis.

In conclusion, chronic administration of a selective organic inhibitor of GSK-3 to the obese Zucker rat, a model of insulin resistance and pre-diabetes, improves oral glucose tolerance and ameliorates whole-body insulin resistance. These metabolic improvements are associated with a diminution of dyslipidemia, an enhancement of insulin-stimulated glucose transport in skeletal muscle, and increases in IRS-1-dependent insulin signaling in skeletal muscle. These results provide further evidence that selective

targeting of GSK-3 in muscle may be an effective intervention in obesity-associated insulin resistance.

CHAPTER 4

ROLE OF GLYCOGEN SYNTHASE KINASE-3 IN OXIDANT STRESS-INDUCED INSULIN RESISTANCE IN SKELETAL MUSCLE

Abstract

The deleterious actions of reactive oxygen species on physiological systems, termed oxidative stress, includes a substantial reduction of insulin action to engage elements of the insulin signaling cascade and to enhance glucose transport activity in skeletal muscle. We have recently found that oxidative stress also decreases the ability of insulin to phosphorylate and thereby suppress the activity of glycogen synthase kinase-3 (GSK-3), a serine/threonine kinase known to inhibit IRS-1-dependent insulin action in skeletal muscle. The present investigation was designed to assess the role of this GSK-3 overactivity in the *in vitro* induction of skeletal muscle insulin resistance by an oxidant stress. Type 1 soleus muscles from insulin-sensitive lean Zucker rats were incubated for 2 hr with or without a maximally-effective concentration of insulin (5 mU/ml) in the absence or presence of glucose oxidase (100 mU/ml), which produces ~90 μM of the oxidant hydrogen peroxide. In the presence of this oxidant stress, insulin-stimulated glucose transport, glycogen synthesis, and glycogen synthase activity were reduced by ~50% ($p<0.05$). Moreover, the oxidant stress also reduced the ability of insulin to induce GSK-3ß ser9 phosphorylation by 52%, allowing GSK3 to retain a more active form. In the presence of CT118637, a selective competitive GSK-3 inhibitor, the ability of insulin to stimulate glucose transport was enhanced by 20% ($p<0.05$) and insulin stimulation of glycogen synthesis was increased by 39% ($p<0.05$). These alterations in insulin-stimulated glucose metabolism in the presence of the GSK3 inhibitor were associated with similar increases of glycogen synthase activity and GSK3ß ser9 phosphorylation. In summary, oxidative stress induces substantial insulin resistance of skeletal muscle

glucose transport and glycogen synthesis. A portion of this oxidative stress-induced insulin resistance is associated with a reduced insulin-mediated suppression of GSK-3 activity. However, there are clearly other cellular mechanisms involved in the deleterious effects of an oxidant stress on the insulin-dependent glucose transport system in skeletal muscle.

Introduction

Skeletal muscle insulin resistance, a major physiologic defect in both pre-diabetes and overt type 2 diabetes [48;124], refers to decreased insulin-stimulated glucose transport and is caused by the inability of insulin to properly signal through an intracellular phosphorylation cascade involving multiple target proteins. Abnormalities in both protein expression and functionality have been shown to contribute to defective insulin signaling. Specifically, insulin-stimulated GLUT-4 protein translocation is diminished in animal models of pre-diabetes and in humans both at risk for and with overt type 2 diabetes [33;65;124]. This decrease in translocation results in diminished glucose transport activity into skeletal muscle [23;33;52;123;124]. Normal translocation of GLUT-4 protein to the plasma membrane in skeletal muscle cells depends on tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) and the docking of IRS-1 to the p85 regulatory subunit of phosphatidylinositol-3' kinase (PI3-kinase). Defects in both of these signaling events in the insulin-resistant state have been well described [4;12;29;40;56;97].

Glycogen synthase kinase-3 (GSK-3) is a serine/threonine kinase that was initially identified as the enzyme that phosphorylates and de-activates glycogen synthase [84;92;122]. GSK-3 consists of highly homologous α and β isoforms [117], is constitutively active, and acutely de-activated by the phosphorylation of specific serine residues on the enzyme. This phosphorylation is mediated by insulin signaling through the sequential activation of IRS-1, PI3-kinase, and ultimately, the kinase activity of Akt [24]. Since its early discovery as a regulator of glycogen synthase activity, GSK-3 has

been shown to modulate many other signaling pathways. Relevant to insulin resistance, GSK-3 activity can result in the phosphorylation of IRS-1 on serine residues, which results in a diminution of insulin signaling [31]. Thus, GSK-3 appears to be a negative modulator of insulin action, resulting in a decrease in glucose transport into skeletal muscle as well as in glycogen synthase activity.

In rodents with pre-diabetes and type 2 diabetes and in type 2 diabetic humans, GSK-3 has been shown to be over-expressed and over-active [29;78], and this overactivity is related to inadequate inhibition by insulin [32;79;79]; reviewed in [30;49;110]). In animal models of obesity-associated insulin resistance, acute pharmacologic inhibition of GSK-3 activity results in lower blood glucose levels, improved whole-body glucose tolerance and insulin-sensitivity [29;49;53;91;110]. Chronic in vivo GSK-3 inhibition in obese pre-diabetic rats also significantly improved whole-body insulin-sensitivity, decreased plasma free fatty acids, and also was associated with marked improvements in the functionality of key insulin signaling proteins, namely, tyrosine phosphorylation of IRS-1 and the docking of PI3-kinase to IRS-1 [29]. Acute in vitro treatment of skeletal muscle from pre-diabetic rats also improved glucose uptake, increased glycogen synthase activity, and was associated with improved IRS-1-dependent insulin signaling.

In recent years, mounting evidence has suggested that oxidative stress can contribute to the multi-factorial etiology of insulin resistance. Oxidative stress occurs when the cellular production of reactive oxygen species (ROS) exceeds the capacity of anti-oxidant defenses within cells. A number of studies have demonstrated increased

oxidative stress in insulin-resistant humans and animals, purportedly related to the excessive metabolism of glucose and free fatty acids present in this physiologic state [81], as well as to the mitochondrial dysfunction associated with insulin resistance [86]. For example, plasma levels of hydroperoxides (ROS) are higher in subjects with type 2 diabetes compared to nondiabetic subjects, and these levels are inversely correlated with the degree of metabolic control [81]. Obesity alone is also associated with increased oxidative stress. In obese, non-diabetic subjects, lipid peroxidation (a marker of oxidant stress) directly correlates with waist circumference and body mass index (BMI), and inversely correlates with adiponectin, an insulin-sensitizing adipokine. [37]. In addition, prolonged exposure of 3T3-L1 adipocytes and L_6 myocytes to H_2O_2 (ROS) significantly attenuates insulin-stimulated glucose metabolism by interfering with normal insulin signaling through PI3-kinase [95]. Taken together, these data suggest that fat accumulation itself could increase oxidative stress in the absence of hyperglycemia, and that increased oxidative stress in obesity might relate to the dysregulated production of adipocytokines as well as other mechanisms that contribute to insulin resistance.

The present study was designed to evaluate the role of GSK-3 in the insulin-resistant state of skeletal muscle induced by oxidative stress, including basal and insulin-stimulated glucose uptake, glycogen synthase activity and glycogen synthesis, and critical elements of the insulin signaling cascade. We hypothesize that the deleterious effect of oxidative stress on insulin action in skeletal muscle can, in part, be reversed by the selective inhibition of GSK-3. This partial recovery of insulin action would result in improvements in glucose transport, glycogen synthase activity, and glycogen

biosynthesis in the muscle exposed to oxidative stress and simultaneously treated with the GSK-3 inhibitor, compared to that exposed to oxidative stress alone. In addition, the amelioration of insulin resistance in the treated muscle would be associated with improvements in the functionality of key components of the insulin signaling pathway.

Methods

Animals. Female lean Zucker rats were purchased from Harlan (Indianapolis, IN) at the age of 8-9 wk and treatments were commenced at 10 wk of age. All animals were housed in a temperature-controlled room (20-22°C) with a 12:12-h light-dark cycle (lights on from 7AM 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. At 8:00 A.M., after an overnight food restriction (chow was restricted to 4 g at 5 pm), animals were deeply anesthetized at 8 am with an intra-peritoneal injection of pentobarbital sodium (50 mg/kg), and strips of soleus muscles (~25-40 mg) were prepared for in vitro incubation in the unmounted state. Soleus strips were incubated for 2 hours at 37°C in 3 ml 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 (radioimmunoassay grade, Sigma Chemical), without or with a maximally effective concentration of insulin (5 mU/ml) in the absence or presence of GSK3 inhibitor CT118637 (1 μM), and in the presence or absence of glucose oxidase (100 mU/ml), which produces ~90 μM of the

oxidant hydrogen peroxide. The GSK3 inhibitor CT118637 (kindly provided by Dr. Steve Harrison, Chiron Corporation, Emeryville, CA) is structurally very similar to and has identical pharmacokinetic properties to selective GSK inhibitors used previously by our research group [29;53;91;110], reviewed in [110]. It inhibits both GSK3α and GSK3ß in vitro with K_i values less than 10 nM in an ATP-competitive manner [29;110].

Measurement of Glucose Transport. Following the incubation period, soleus strips were rinsed for 10 min at 37°C in 3 ml of oxygenated KHB containing 40 mM mannitol, 0.1% BSA, and insulin, if previously present. 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/mmol; Sigma Chemical), 39 mM [U-^{14}C] mannitol (0.8 μCi/mmol; ICN Radiochemicals, Irvine, CA), 0.1% BSA, 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, and weighed. These muscles were dissolved in 0.5 ml of 0.5 N NaOH, 5 ml of scintillation cocktail were added, and the specific intracellular accumulation of 2-deoxy-[1,2-^{3}H] glucose was determined as described previously [51]. This method for assessing glucose transport activity in isolated muscle has been validated [44].

Measurement of glucose incorporation into glycogen.

Soleus strips were incubated for 2 hours in oxygenated KHB (as described earlier) with the addition of [U-^{14}C] glucose (0.10 μCi/ml), in the absence and/or presence of maximal insulin stimulation (5 mU/ml), 1 μM CT118637, and/or glucose oxidase (100 mU/ml). Muscle was dissolved in 1 ml of 5N KOH and heated to boiling in a water bath.

Saturated sodium sulfate (0.2 ml) was added to each sample, followed by 1.5 ml of 95% ETOH. Samples were again heated to boiling for 1-2 minutes, then cooled. The homogenate was centrifuged at 2000g for 10 minutes, during which time the glycogen accumulates as a pellet. The glycogen pellets were dissolved in 1 ml of water and heated until they dissolved (1-3 minutes). The ETOH precipitation and centrifugation steps were repeated and the supernatant was removed. The remaining glycogen pellet was dissolved in 0.5 ml of water. The samples were transferred to scintillation vials, scintillation cocktail was added (5 ml) and activity in the ^{14}C channel was assessed.

Measurement of glycogen synthase activity. Glycogen synthase activity in soleus 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 [106].

Assessment of insulin signaling factor protein expression and functionality. Frozen tissues were homogenized in 8 vol of ice-cold lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 20 mM Na pyrophosphate, 20 mM ß-glycerophosphate, 10 mM NaF, 2 mM Na_3VO_4, 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 PMSF). Homogenates were incubated on ice for 20 min and then centrifuged at 13,000 X g for 20 minutes at 4°C. Total protein concentration was determined using the BCA method (Sigma Chemical). Insulin signaling proteins were separated by SDS-PAGE on 7.5% or 12% polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA) and transferred to nitrocellulose membranes. To determine the functionality of insulin signaling factors, the

blots were incubated with the commercially available antibodies against phospho-Akt ser^{473} and phospho-GSK3α/ß ser$^{21/9}$ (Cell Signaling Technology) overnight. Membranes were then incubated with secondary goat anti-rabbit antibody conjugated with horse-radish peroxidase (HRP) (Chemicon, Temecula, CA). The proteins were visualized on Kodak X-Omat AR film (Kodak, Rochester, NY) using an enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia, Piscataway, NJ). The band intensities on the autoradiographs were quantified on a scanning densitometer (Bio-Rad Model GS-800) using Quantity One software (Bio-Rad).

For measurement of tyrosine-phosphorylated IRS-1 (IRS-1/pY) and for IRS-1 associated p85 (IRS-1/p85), immunoprecipitations and subsequent immunoblotting were performed. For analysis of IRS-1/pY, 0.5 ml of diluted homogenate (1 µg/µl) was immunoprecipitated with 25 µl of agarose-conjugated anti-IRS-1 antibody (Upstate Biotechnology). For analysis of IRS-1/p85, 0.5 ml of diluted homogenate (1 µg/µl) was immunoprecipitated with 25 µl of agarose-conjugated anti-p85 antibody (Upstate Biotechnology). After an incubation period of 4 hours for IRS-1/p85, and overnight incubation at 4°C for IRS-1/pY, samples were centrifuged and the supernatant was removed. The beads were washed three times with ice-cold PBS, mixed with SDS sample buffer, and boiled for 5 minutes. Proteins were separated by SDS-PAGE on 7.5% polyacrylamide gels (Bio-Rad Laboratories) and transferred to nitrocellulose membranes. For analysis of IRS-1/pY, the nitrocellulose membrane was incubated in anti-phosphotyrosine antibody (PY99, Santa Cruz Biotechnology, Santa Cruz, CA). Immunoblotting for detection of IRS-1/p85 was completed as described above using an

antibody against IRS-1 (Upstate Biotechnology) 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. For metabolic assays, differences between two groups were determined using an unpaired Student's *t*-test. For insulin signaling data, differences between two groups were determined using a paired Student's *t*- test (muscles from the same animal) or unpaired Student's *t*- test (when comparing muscles from different animals) .A level of $P< 0.05$ was set for statistical significance.

Results

Effect of oxidant stress. Isolated soleus muscle strips from lean Zucker rats were subjected to treatment with glucose oxidase (100 mU/ml), which produces ~90 μM of the oxidant hydrogen peroxide. The oxidant stress increased basal glucose transport by 26% ($p<0.05$, fig.15 A). Accordingly, basal glycogen synthesis (glucose incorporation into glycogen) was increased by 29% ($p<0.05$, fig. 15 B) in the presence of the oxidant stress. The oxidant stress also increased the activity of glycogen synthase by 30% ($p<0.05$, fig. 15 C). In the basal state, oxidative stress did not change the phosphorylation state of GSK-3 or Akt (data not shown; Akt bands not detected).

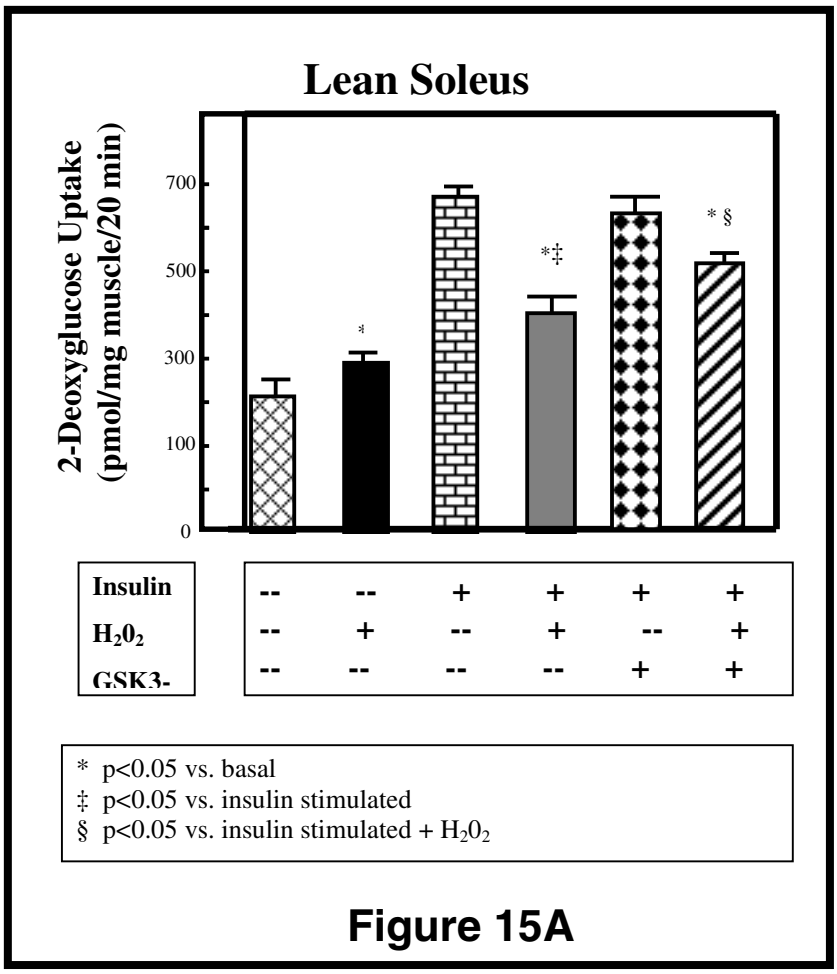


Figure 15A

Figure 15A. Effects of an oxidant stress (H_20_2) and/or GSK3 inhibitor CT118637 (1 µM) on basal or insulin-stimulated glucose transport activity in skeletal muscle of lean Zucker rats. Soleus strips were incubated for 120 min without or with insulin (5 mU/ml) and without or with glucose oxidase (100 mU/ml), which produces ~90 µM of the oxidant hydrogen peroxide. 2-Deoxyglucose uptake was then performed as described in METHODS. Values are means ± SE for 4-5 muscles per group. *P<0.05 vs. basal, ‡ P<0.05 vs. insulin stimulated, § P<0.05 vs. insulin stimulated + H_20_2.

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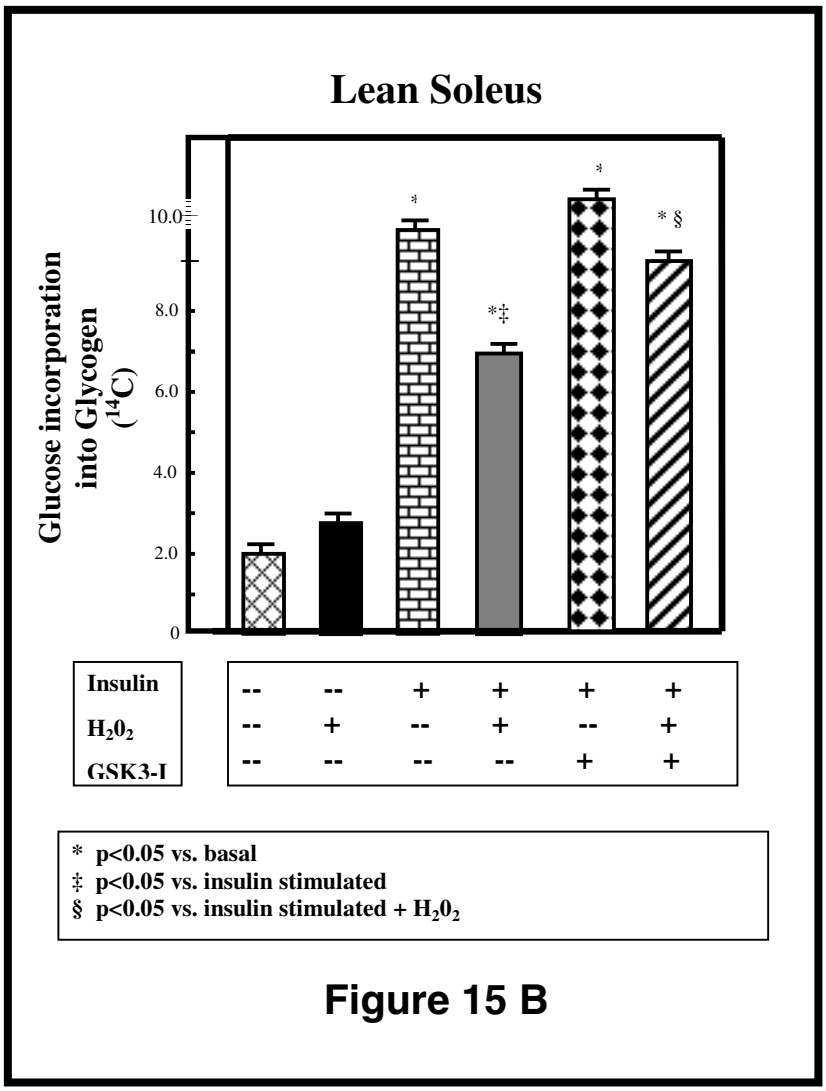

Figure 15 B

Figure 15B. Effects of an oxidant stress (H_2O_2) and/or GSK3 inhibitor CT118637 (1 µM) on basal or insulin-stimulated glycogen synthesis in skeletal muscle of lean Zucker rats. Soleus strips were incubated for 120 min without or with insulin (5 mU/ml) and without or with glucose oxidase (100 mU/ml), which produces ~90 µM of the oxidant hydrogen peroxide. Glucose incorporation into glycogen was then performed as described in METHODS. Values are means ± SE for 4-5 muscles per group. *P<0.05 vs. basal, ‡ P<0.05 vs. insulin stimulated, § P<0.05 vs. insulin stimulated + H_2O_2.

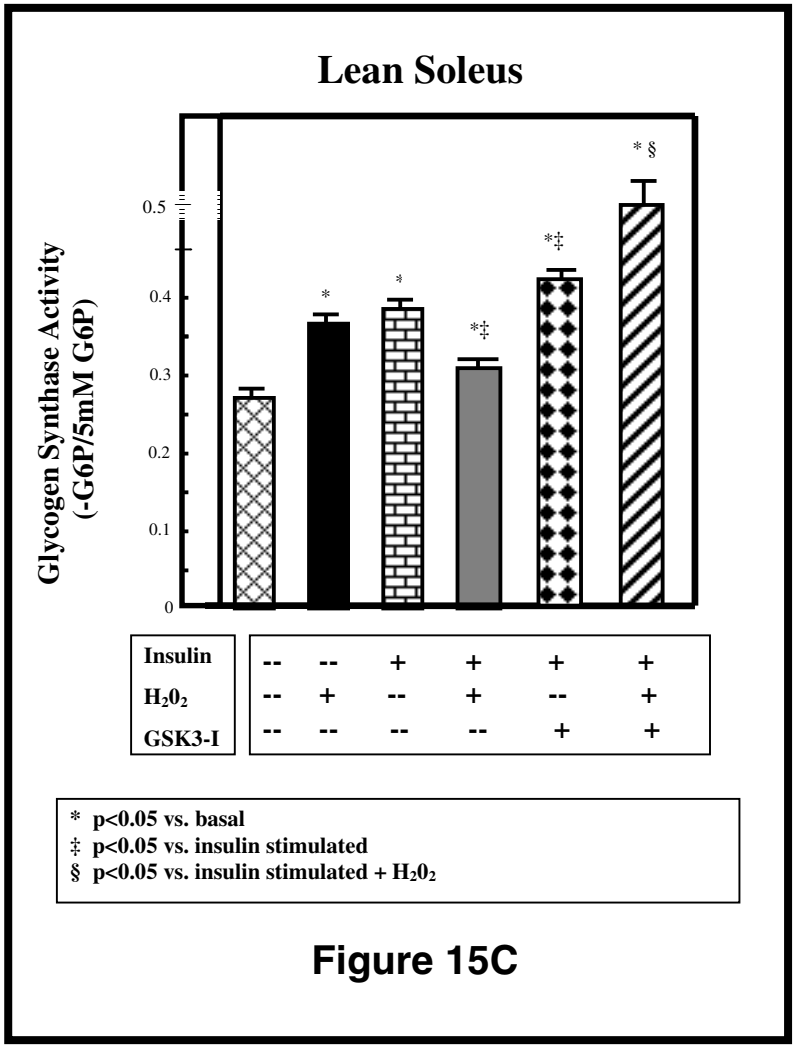

Figure 15C. Effects of an oxidant stress (H_2O_2) and/or GSK3 inhibitor CT118637 (1 µM) on basal or insulin-stimulated glycogen synthase activity in skeletal muscle of lean Zucker rats. Soleus strips were incubated for 120 min without or with insulin (5 mU/ml) and without or with glucose oxidase (100 mU/ml), which produces ~90 µM of the oxidant hydrogen peroxide. The glycogen synthase activity assay was then performed as described in METHODS. Values are means ± SE for 4-5 muscles per group. **P*<0.05 vs. basal, ‡ *P*<0.05 vs. insulin stimulated, §*P* <0.05 vs. insulin stimulated + H_2O_2.

Effect of oxidant stress on insulin-stimulated muscle. To assess potential relationships between insulin and oxidative stress in skeletal muscle, we incubated

isolated soleus muscle strips from lean Zucker rats in the presence or absence of glucose oxidase, and with or without a maximally effective concentration of insulin (5 mU/ml). Compared to basal conditions, insulin alone markedly increased glucose transport (217% $p<0.05$, fig. 15A), glycogen synthesis (363%, $p<0.05$, fig. 15B), and glycogen synthase activity ratio (38% $p<0.05$, fig 15C).

Oxidant stress had deleterious effects on insulin action in skeletal muscle from lean Zucker rats. In the presence of the oxidant stress, insulin-stimulated glucose transport (40%, fig. 15A), glycogen synthesis (31%, fig. 15B), and glycogen synthase activity (5%, fig. 15C) were significantly reduced ($p<0.05$). The oxidant stress also reduced the ability of insulin to induce GSK-3ß ser^{9} phosphorylation by 49% ($p<0.05$, fig. 16A), allowing GSK3 to retain a more active form. In addition, insulin-stimulated Akt ser^{473} phosphorylation was 62% lower in the muscles exposed to the oxidant stress ($p<0.05$, fig. 16B), indicating a substantial diminution of the activity of this key insulin signaling protein. Oxidative stress had no detectable effect on insulin-stimulated tyrosine phosphorylation of IRS-1 or or IRS1-associated p85 (data not shown).

Effect of selective GSK-3 inhibition on oxidant stress-induced insulin resistance. In the presence of the oxidant stress, the selective GSK-3 inhibitor CT118637 partially reversed the deleterious effects of the oxidative stress on insulin action. CT118637 (1 μM) increased insulin-stimulated glucose transport by 20% ($p<0.05$, fig 15A), glycogen synthesis by 35% ($p<0.05$, fig. 15B), and glycogen synthase activity by 42% ($p<0.05$, fig. 15C) over that observed in insulin-stimulated muscles exposed to oxidant stress alone. There was no difference in glucose transport with the addition of CT118637 to insulin

alone. Glycogen synthase activity was actually enhanced above the level of insulin stimulation alone. In addition, the selective GSK-3 inhibition increased insulin-stimulated GSK3ß ser^{9} phosphorylation by 152% ($p<0.05$, fig. 16A) over that observed in the muscles subjected to oxidative stress, and Akt ser^{473} phosphorylation was enhanced by 164% ($p<0.05$, fig. 16B) above that observed with the oxidant stress. No change was observed in insulin-stimulated tyrosine phosphorylation of IRS-1 or IRS-1-associated PI3-kinase (data not shown).

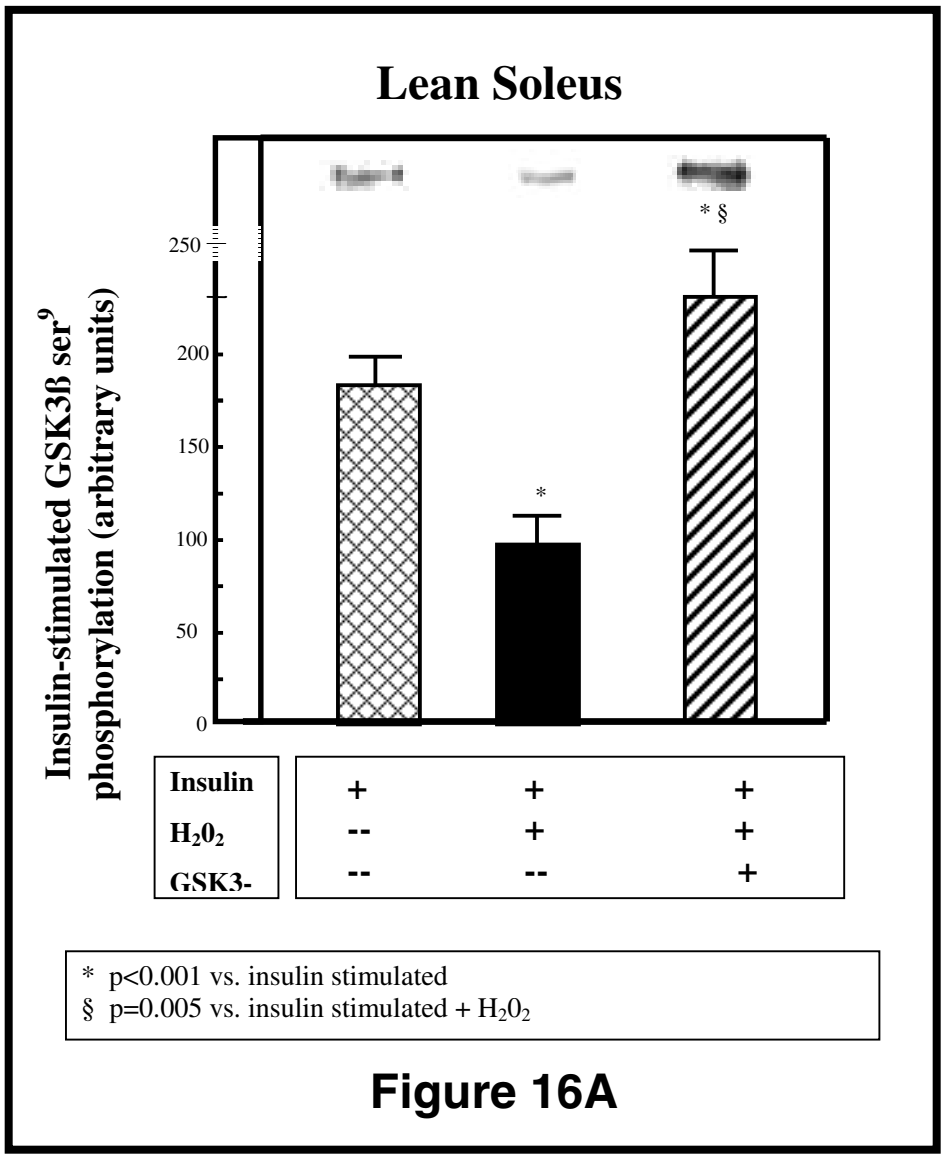

Figure 16A

Figure 16A. Effects of an oxidant stress (H_20_2) and/or GSK3 inhibitor CT118637 (1 μM) on GSK-3□ serine[9] phosphorylation in type I skeletal muscle of lean Zucker rats. Soleus strips were incubated for 120 min without or with insulin (5 mU/ml) and without or with glucose oxidase (100 mU/ml), which produces ~90 μM of the oxidant hydrogen peroxide. Immunoblotting was then performed as described in METHODS. Values are means ± SE for 4-5 muscles per group. *P<0.05 vs. basal, ‡P<0.05 vs. insulin stimulated, §P<0.05 vs. insulin stimulated + H_20_2.

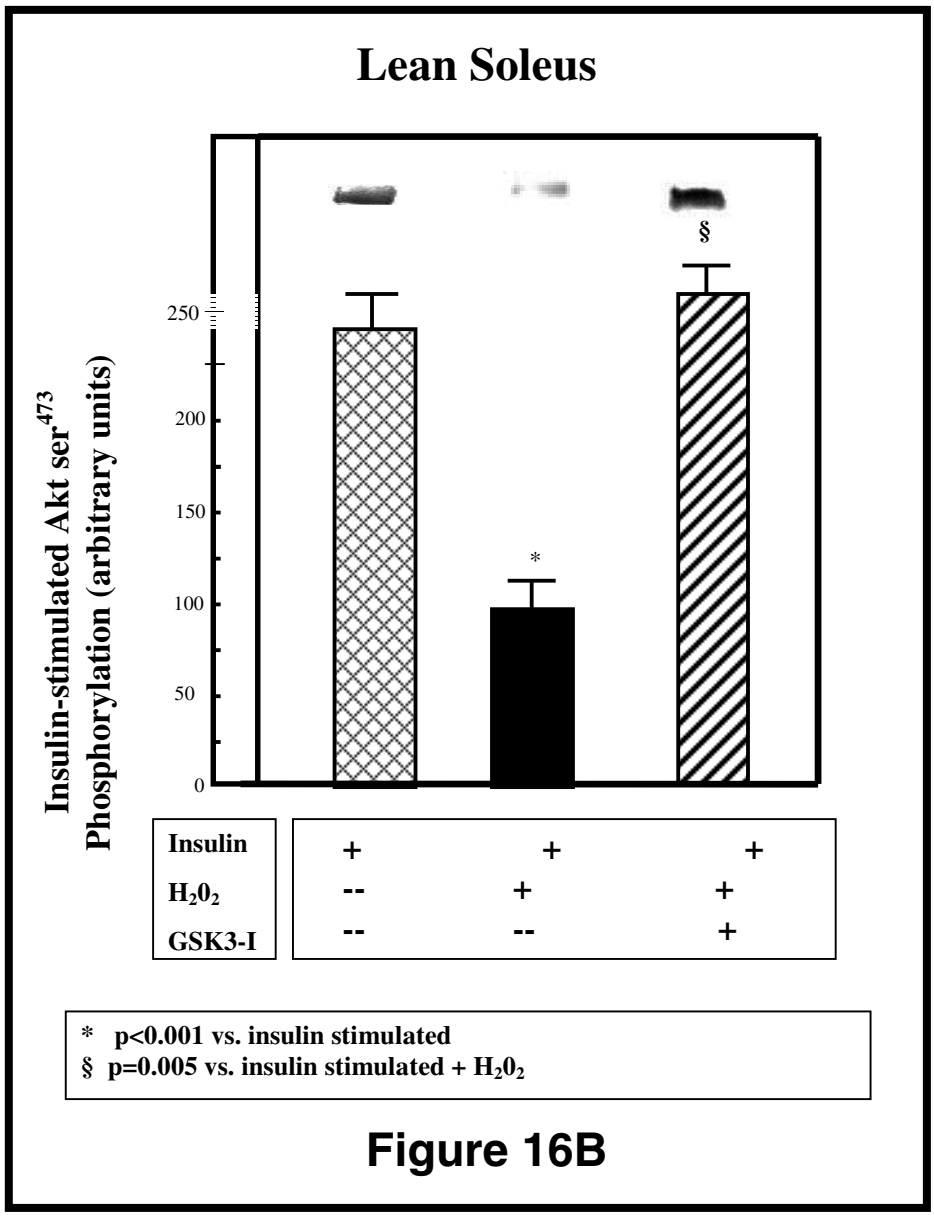

Figure 16B

Figure 16B. Effects of an oxidant stress (H_20_2) and/or GSK3 inhibitor CT118637 (1 μM) on Akt serine^{473} phosphorylation in type I skeletal muscle of lean Zucker rats. Soleus strips were incubated for 120 min without or with insulin (5 mU/ml) and without or with glucose oxidase (100 mU/ml), which produces ~90 μM of the oxidant hydrogen peroxide. Immunoblotting was then performed as described in METHODS. Values are means ± SE for 4-5 muscles per group. *P<0.05 vs. basal, ‡P<0.05 vs. insulin stimulated, §P<0.05 vs. insulin stimulated + H_20_2.

Discussion

The results of the present investigation demonstrate the novel finding that acute, selective inhibition of GSK-3 with CT118637 can partially mitigate the skeletal muscle insulin resistance induced by oxidative stress in lean, insulin-sensitive obese Zucker rats. These beneficial effects of GSK-3 inhibition on insulin action are manifested in a higher rate of glucose transport, as well as enhanced glycogen synthase activity and glycogen synthesis (glucose incorporation into glycogen). Additional novel findings include improved insulin-stimulated activation of Akt and markedly enhanced de-activation of deleterious GSK-3.

In insulin-stimulated skeletal muscle exposed to an oxidant stress, selective inhibition of GSK-3 resulted in a significant improvement in insulin action over the oxidant stress alone; however, it did not lead to the complete recovery of normal insulin action. Multiple mechanisms have been proposed to explain oxidative stress-induced insulin resistance (reviewed in ref. [14]). Blair [13] observed that oxidant stress-induced insulin resistance in L6 myotubes can be rapidly reversed, which suggests that oxidant stress modulates insulin action through its signaling pathway [13]. Oxidative stress has been shown to induce IRS1 serine phosphorylation and impair insulin signaling, and a number of serine kinases could be implicated [14]. Rudich, et al [95] showed that H_2O_2 significantly attenuated insulin-stimulated glucose transport and translocation of GLUT-4 to plasma membranes, an effect associated with decreased PI3-kinase and Akt activation and increased IRS-1 serine phosphorylation and degradation [95]. In vascular smooth muscle cells, H_2O_2 decreased autophosphorylation of the insulin receptor and subsequent

phosphorylation of Akt [39]. In addition, in skeletal muscle, oxidative stress impaired the ability of insulin to stimulate the phosphorylation and de-activation of GSK-3 [96].

GSK-3 is a serine/threonine kinase, and is able to phosphorylate IRS-1 on serine residues [31] and subsequently impair insulin action. The present study suggests that oxidative stress impairs insulin signaling, at least in part, by de-activating Akt, a critical element in the signaling pathway leading to the insulin-dependent translocation of GLUT 4 to the sarcolemmal membrane; the key step in the process of glucose uptake into skeletal muscle. Activated Akt is known to target GSK-3, whose serine phosphorylation results in its de-activation; a crucial step in the activation of glycogen synthase. Consistent with our findings of impaired activity of Akt is the decreased level of phosphorylation of GSK-3, which indicates its over-activity in response to oxidative stress. Taken together, these data suggest that oxidative stress impairs the ability of insulin to activate Akt, and consequently, GSK-3 remains in a more de-phosphorylated, active state.

In the present study, insulin-stimulated glycogen synthase activity and glycogen synthesis were both impaired by oxidative stress. Insulin action was recovered (partially in the case of glycogen synthesis, and in excess of insulin in the case of glycogen synthase activity) with the addition of CT11637. Insulin-stimulated glucose transport was also decreased by oxidative stress and partially recovered with selective GSK-3 inhibition.

In conclusion, the present study demonstrates that selective GSK-3 inhibition leads to the partial recovery of insulin action that was diminished by oxidative stress, and

this action of the GSK-3 inhibtion was associated with enhanced serine phosphorylation of Akt and GSK-3ß. However, there are clearly other cellular mechanisms involved in the deleterious effects of oxidant stress on the insulin-dependent glucose transport system in skeletal muscle.

CHAPTER 5

SUMMARY OF MAJOR FINDINGS

The metabolic syndrome is a multifaceted condition characterized by a clustering of metabolic and cardiovascular abnormalities, including insulin resistance in skeletal muscle, adipose tissue and liver; visceral adiposity, hyperinsulinemia, glucose intolerance, dyslipidemia, and essential hypertension. In the United States, the metabolic syndrome has reached epidemic proportions [17]. Those affected by this syndrome are at very high risk for developing type 2 diabetes and all of the related sequelae, such as blindness, kidney failure, amputation, and particularly cardiovascular disease.

Glycogen synthase kinase-3 (GSK-3) is a serine/threonine kinase involved in the regulation of glucose metabolism through the phosphorylation and de-activation of glycogen synthase [84]. It also contributes to the multi-factorial etiology of insulin resistance by attenuating insulin signaling in skeletal muscle, thereby decreasing GLUT-4 translocation and subsequent glucose uptake [31]. GSK-3 is known to be overactive in humans with type 2 diabetes, and in animal models of both type 2 diabetes and the metabolic syndrome (pre-diabetes) [32].

In recent years, mounting evidence has suggested that one additional factor, oxidative stress, can contribute to the multi-factorial etiology of insulin resistance. Oxidative stress occurs when the cellular production of reactive oxygen species (ROS) exceeds the level of anti-oxidant defenses within cells. A number of studies have demonstrated increased oxidative stress in insulin-resistant humans and animals, which may be related to mitochondrial dysfunction and/or to excess available sustrate for metabolic processes [49].

This study was undertaken to determine the role of GSK-3 in obesity-associated insulin-resistance, and further, to elucidate the role of GSK-3 in the insulin-resistant state induced by oxidative stress. The genetically obese Zucker rat (*fa/fa*) is a model of insulin resistance characterized by hyperinsulinemia, glucose intolerance, obesity, dyslipidemia, and insulin resistance of skeletal muscle. For the first two phases of this study, the obese Zucker rat was used as a model of obesity associated insulin resistance and the metabolic syndrome. Using the obese Zucker rat, we determined the effects of selective GSK-3 inhibition on obesity-associated insulin resistance, first in isolated skeletal muscle, and then chronically treated the animals with the GSK-3 inhibitor CT118637 *in vivo* to investigate the whole-body responses. In the final phase of this study, Zucker lean control rats were used as a model of insulin sensitive animals. H_2O_2 was produced in isolated skeletal muscle from these animals in order to create a model of ROS-induced insulin resistance. Again, using isolated skeletal muscle, we elucidated the effects of GSK3, and its selective inhibition, on insulin resistance and insulin action.

Initially, type I soleus and type IIb epitrochlearis muscles from female obese Zucker rats were incubated in the absence or presence of a selective, small organic GSK-3 inhibitor (1 μM CT118637) and in the absence or presence of maximal insulin stimulation (5 mU/ml). Glucose transport activity, glycogen synthase activity, and key insulin signaling factors (tyrosine phosphorylation of insulin receptor (IR) and IRS-1, IRS-1 associated with p85 subunit of phosphatidylinositol-3-kinase, and serine phosphorylation of Akt and GSK-3) were assessed.

Acute *in vitro* treatment of isolated skeletal muscle from insulin-resistant, pre-diabetic female obese Zucker rats with the selective GSK-3 inhibitor CT118637 potentiated the ability of insulin to stimulate both glycogen synthase activity and glucose transport activity. Although GSK-3 inhibition was apparent in all muscles, insulin action on glucose transport activity was enhanced only in insulin-resistant obese muscles, and selective inhibition of GSK-3 had no effect on glucose transport activity in insulin-sensitive skeletal muscles from lean Zucker rats. These data support the hypothesis, and the findings of others, that elevated GSK-3 activity, which exists in insulin-resistant muscle, is necessary for improvement of glucose transport activity by GSK-3 inhibition.

We also found 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, a critical element of the insulin signaling cascade in muscle, and, as previously discussed, a protein that can be negatively modulated by GSK-3. The enhancement of insulin action mediated by the GSK-3 inhibitor was clearly distal to the insulin receptor (IR) in obese

skeletal muscle, as a similar GSK-3 inhibitor-mediated upregulation of insulin action was not observed for tyrosine phosphorylation of the IR ß-subunit. The enhanced insulin-stimulated IRS-1 tyrosine phoshosphorylation induced by the GSK-3 inhibitor had important downstream effects in the insulin signaling cascade, as insulin action on IRS-1 associated with the p85 subunit of PI3-kinase, ser^{473} phosphorylation on Akt, and ser^{9} phosphorylation of GSK-3ß were similarly upregulated in skeletal muscle from the obese Zucker rat incubated with the selective GSK-3 inhibitor.

These data support a mechanistic connection between the upregulation of Akt serine phosphorylation and GSK-3ß serine phosphorylation and the potentiation of insulin stimulation of glycogen synthase activity due to the GSK-3 inhibition. The enhanced insulin-stimulated Akt activity (as reflected by the increased serine phosphorylation state) would catalyze greater GSK-3 phosphorylation, and lead to less GSK-3 activity, thereby further decreasing the ability of GSK-3 to phosphorylate and deactivate glycogen synthase, a direct target of GSK-3 action.

The effects of the GSK-3 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 compared to the type IIb epitrochlearis muscle of the obese Zucker rat. One factor that may underlie this differential response to GSK-3 inhibition is the fact that the protein expression and/or functionality of the insulin signaling factors of interest, including IRS-1 tyrosine phosphorylation, phosphotyrosine associated PI3-kinase activity, and Akt ser^{473} phosphorylation, are greater in type I muscle compared to type IIb muscle, and [100] the protein expression of GLUT4 is likewise greater in type I

soleus muscle than in type IIb epitrochlearis muscle. 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, and therefore one would expect that an intervention, such as selective GSK-3 inhibitors, that acts on the functionality of the insulin signaling elements would invoke a more robust response in type I muscle compared to type IIb muscle.

Chronic*, in vivo* treatment of obese Zucker rats with CT118637 improved glucose tolerance and whole-body insulin sensitivity. The obese Zucker rats treated chronically with the selective GSK-3 inhibitor had significantly lower fasting insulin levels in the presence of plasma glucose levels that were similar in both groups, indicating that less insulin was required to regulate the plasma glucose level (greater insulin sensitivity). In addition, the glucose response to the oral glucose load was markedly reduced in the chronic GSK-3 inhibitor-treated group at the 15-min and 30-min time points and remained reduced up to 120 min. The insulin response was likewise blunted in the chronic GSK-3 inhibitor-treated group, with a significant reduction at the 15-min time point. The total area under the glucose curve (AUC_g) was significantly reduced by the chronic GSK-3 inhibitor treatment. The whole-body insulin sensitivity index, reflecting insulin sensitivity, was substantially enhanced in the chronic GSK-3 inhibitor-treated group. In addition, the maximal capacity for insulin-stimulation of glucose transport activity was upregulated in type I soleus and type II epitrochlearis muscles of the chronic GSK-3 inhibitor-treated obese animals.

Because the chronic oral treatments with the selective GSK-3 inhibitor caused a significant decrease in plasma FFA levels, it is possible that the improvement in the lipemic state of the obese Zucker rats is mechanistically associated with the enhanced whole-body insulin sensitivity in the treated animals. It is known that FFAs or their derivatives can directly and indirectly inhibit the functionality of IRS-1-dependent insulin signaling in skeletal muscle [121]. Therefore, a decrease in the plasma levels of these inhibitory lipids following chronic GSK-3 inhibitor treatment could allow for greater signaling via IRS-1-dependent steps, such as PI3-kinase, and a similar enhancement of muscle glucose disposal.

The enhancement of whole-body insulin sensitivity in the fasting state, elicited in the chronically treated obese Zucker rats, was also associated with significant improvements in the functional status of key elements of the insulin signaling cascade in muscle, namely tyrosine phosphorylation of IRS-1 and IRS-1 association with the p85 subunit of PI3-kinase. Moreover, the chronic GSK-3 inhibition upregulated the functionality of IRS-1 and PI3-kinase in muscle in the absence of any significant alterations in the protein expression of a wide variety of insulin signaling factors and GLUT-4.

Acute, selective GSK-3 inhibition also had beneficial effects on insulin action in isolated, insulin sensitive skeletal muscle from lean Zucker rats in which insulin resistance was induced *in vitro* by oxidant stress. These effects are manifested in a higher rate of glucose transport, as well as enhanced glycogen synthase activity and glycogen synthesis (glucose incorporation into glycogen). Oxidant stress resulted in decreased

insulin action, and in insulin-stimulated skeletal muscle exposed to an oxidant stress, selective inhibition of GSK-3 resulted in a significant improvement in insulin action over the oxidant stress alone. However, the inhibition of GSK-3 did not lead to the complete recovery of normal insulin action. Multiple mechanisms have been proposed to explain oxidative stress-induced insulin resistance, most likely mediated via impairment of normal insulin signaling. Oxidative stress has been shown to induce IRS1 serine phosphorylation and impair insulin signaling. GSK-3 is one of several kinases able to phosphorylate IRS-1 on serine residues and subsequently impair insulin action.

The activation states of a variety of key insulin signaling proteins was determined: IRS1, PI3-kinase, Akt, and GSK-3. Although no differences were found between treatment groups in IRS1 tyrosine phosphorylatioin or IRS1-association with PI3-kinase, marked differences were found in Akt and GSK-3. Oxidant stress significantly decreased the level of insulin-stimulated phosphorylation of Akt, rendering it less active in these muscles. However, the addition of CT118637 to insulin resulted in an increased phosphorylation, albeit not to the level of insulin alone. The insulin-stimulated phosphorylation of GSK-3 was also severely attenuated by oxidant stress, however, muscles simultaneously treated with the GSK-3 inhibitor exhibited a level of phosphorylation over and above that of insulin stimulation alone.

In addition, insulin-stimulated glycogen synthase activity and glycogen synthesis were both impaired by oxidative stress, and the addition of CT11637 resulted in a recovery (partially in the case of glycogen synthesis, and in excess of insulin in the case of glycogen synthase activity) of insulin action on these metabolic processes. Insulin-

stimulated glucose transport was also decreased by oxidative stress and partially recovered with selective GSK-3 inhibition.

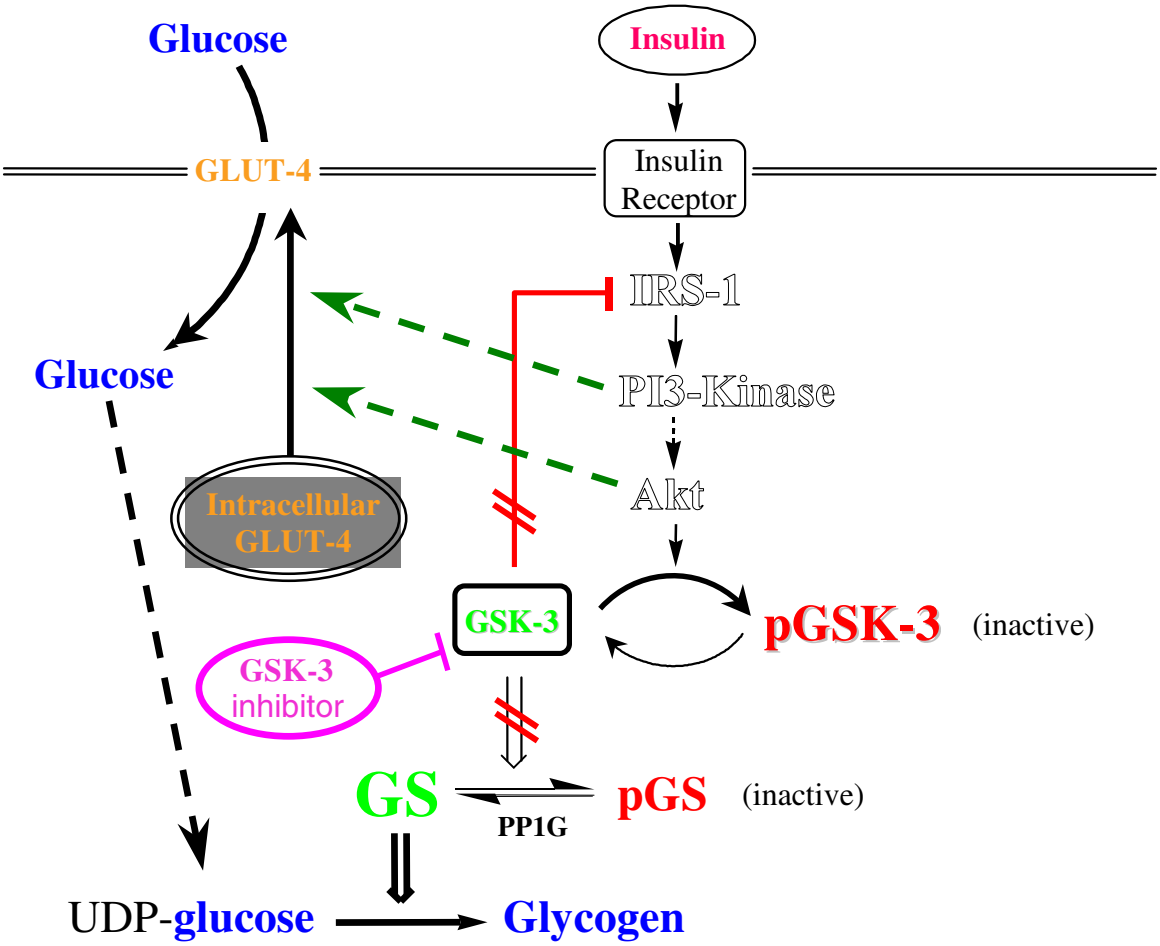

FIGURE 17

Figure 17. Effects of GSK-3, and its selective inhibition, on key steps in the insulin signaling pathway.

In summary (fig. 17), obesity-associated insulin resistance as well as that induced by oxidative stress can be mitigated by selective GSK-3 inhibition. The etiology of

insulin resistance is multi-factorial, and results in the attenuation of glucose transport, glycogen synthase activity, and glycogen synthesis, as well as the regulation of the plasma glucose concentration. In each of the models investigated in this study, selective GSK-3 inhibition resulted in a partial amelioration of the insulin-resistant state. At the cellular level, insulin resistance is demonstrated by impaired insulin signaling, mediated by the poorly regulated activity of key proteins involved in the translocation of GLUT-4 transporters to the plasma membrane. Again, in each of the models utilized in this study, selective GSK-3 inhibition was shown to be associated with improved activation and function of key insulin signaling factors.

Taken together, these results provide convincing evidence that selective targeting of GSK-3 in muscle may be an effective intervention in obesity-associated, and ROS-associated insulin resistance.

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