Published in final edited form as:

Mol Cell Endocrinol. 2010 February 5; 315(1-2): 153. doi:10.1016/j.mce.2009.05.020.

GSK-3 β and Control of Glucose Metabolism and Insulin Action in Human Skeletal Muscle

T. P. Ciaraldi, L. Carter, S. Mudaliar, and R. R. Henry

Veterans Affairs San Diego Healthcare System and Department of Medicine, University of California, San Diego

Summary

The involvement of the β -isoform of Glycogen Synthase Kinase (GSK-3) in glucose metabolism and insulin action was investigated in cultured human skeletal muscle cells. A 60% reduction in GSK-3 β protein expression was attained by treatment with siRNA; GSK-3 α expression was unaltered. GSK-3 β knockdown did not influence total glycogen synthase (GS) activity, but increased the phosphorylation-dependent activity (fractional velocity – FV) in the basal state. Insulin responsiveness of GSFV was doubled by GSK-3 β knockdown ($p < 0.05$). Basal rates of glucose uptake (GU) were not significantly influenced by GSK-3 β knockdown, while insulin stimulation of GU was increased. Improvements in insulin action on GS and GU did not involve changes in protein expression of either IRS-1 or Akt 1/2. Maximal insulin stimulation of phosphorylation of Akt was unaltered by GSK-3 β knockdown. Unlike GSK-3 α , GSK-3 β directly regulates both GS activity in the absence of added insulin and through control of insulin action.

Keywords

Glycogen synthase kinase 3; insulin action; muscle (human skeletal); glucose uptake; IRS-1

Introduction

Glycogen synthase kinase-3 (GSK-3) is a broadly distributed serine/threonine kinase that, in addition to its regulation of glycogen metabolism, plays a role in a number of cellular processes. These include cell growth and differentiation, apoptosis, neural degeneration, and protein synthesis (reviewed in (Woodgett, 2003)). GSK-3 has several uncommon characteristics. One is that its substrates require a priming phosphorylation four residues C-terminal to the GSK3 phosphorylation site (SXXXpS). In addition, GSK-3 is constitutively active, with deactivation occurring in response to serine phosphorylation of the protein itself (Shaw et al., 1997).

GSK-3 is present in two forms, α and β , the products of distinct genes located on chromosomes 19q13.1-2 and 3q13.3-q21, respectively (Hansen et al., 1997). While the forms share 98% amino acid homology in the kinase domain and 85% homology across the entire molecule (Doble and Woodgett, 2003), the α -isoform is a 51-53 kDa polypeptide, and the β -isoform is a 47-kDa polypeptide. The major structural differences include an N-terminal extension in

Corresponding author: Robert R. Henry, M.D. VA San Diego Healthcare System 3350 La Jolla Village Drive San Diego, CA 92161 ph: (858) 552-8585 x3648 fax: (858) 642-6242 rhenry@vapop.ucsd.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

GSK-3 α and variability in the C-terminal region. Inactivating phosphorylation occurs on related sites, serine 21 and serine 9, in $-\alpha$ and $-\beta$, respectively (reviewed in (Doble and Woodgett, 2003). In spite of these similarities, the proteins are not fully functionally interchangeable. Whole animal knockouts of GSK-3 β are embryonically lethal and cannot be rescued by overexpression of GSK-3 α (Hoeftlich et al., 2000). Conversely, GSK-3 α knockout mice are viable (MacAulay et al., 2007). We previously found that acute insulin infusion reduced GSK-3 α activity in human skeletal muscle to a greater extent than that of $-\beta$ and that GSK-3 α activity and protein expression displayed a stronger association with whole body insulin action (Nikoulina et al., 2000).

These differences in regulation may be significant as there is a growing body of evidence that supports an involvement of GSK-3 in insulin resistance. GSK-3 expression ($-\alpha$ and $-\beta$) is elevated in the skeletal muscle of poorly controlled type 2 diabetic subjects (Nikoulina et al., 2000) and adipose tissue of insulin resistant animals (Eldar-Finkelman et al., 1999). Over expression of GSK-3 β , in cultured cells (Summers et al., 1999), and in vivo in skeletal muscle (Pearce et al., 2007), results in insulin resistance for glucose metabolism while a whole body knockout of GSK-3 α displays increased insulin sensitivity (MacAulay et al., 2007). A variety of chemically distinct selective and specific inhibitors of GSK-3 have been shown to improve insulin action, both in vivo in animal models of insulin resistance (Cline et al., 2002; Doken and Henriksen, 2006), and in vitro in tissues (Ring et al., 2003) and cultured cells (Coghlan et al., 2004; Nikoulina et al., 2002). However, these inhibitors do not distinguish between isoforms (reviewed in (Cohen and Goedert, 2004)). We recently manipulated GSK-3 α expression in cultured human skeletal muscle cells and found that overexpression impaired insulin action while reducing expression improved insulin responsiveness, yet neither intervention influenced glucose metabolism in the absence of insulin (Ciaraldi et al., 2007). The current report extends these studies to GSK-3 β , investigating its role in control of skeletal muscle glucose metabolism and insulin action in cultured human muscle cells.

Materials and Methods

Materials

Cell culture materials were purchased from Irvine Scientific (Irvine, CA) except for skeletal muscle basal medium (SkGM), which was obtained from Lonza (Walkersville, MD) and OptiMEM, purchased from GIBCO (Grand Island, NY). Fetal bovine serum was obtained from Gemini Bio-Products (Woodland, CA). BSA (Fraction V) was purchased from Boehringer Mannheim (Indianapolis, IN). siImporter transfection reagent and the GSK3 β siRNA/siAB assay kit, which includes validated pooled (four duplexes) specific siRNA targeting GSK-3 β and non-specific control siRNA, were purchased from Upstate Biotechnology (Charlottesville, VA). Glycogen, 2-deoxyglucose, glucose-6-P, leupeptin, aprotinin, sodium fluoride, sodium pyrophosphate, sodium orthovanadate, phenylmethylsulfonyl fluoride (PMSF), and other reagents and chemicals were purchased from Sigma (St. Louis, MO). L- [14 C] glucose, [3 H]-2-deoxyglucose, and [14 C]-UDP-glucose, were obtained from Perkin Elmer NEN (Boston, MA). Protein assay reagents and electrophoresis chemicals were purchased from Bio-Rad Laboratories (Hercules, CA). Anti-GSK-3/Shaggy protein kinase family (51/46-kDa) mouse monoclonal IgG, which recognizes an epitope common to GSK-3 α and $-\beta$, and anti-rat C-terminal IRS-1 rabbit polyclonal IgG were purchased from Upstate Biotechnology (Charlottesville, VA). Anti-pS⁴⁷³- and pT³⁰⁸-Akt antibodies were purchased from Cell Signaling Technology (Beverly, MA) while anti-Akt1/2 was from Santa Cruz Biotechnology (Santa Cruz, CA). A polyclonal antibody against human GS was a kind gift of Dr. John Lawrence (Charlottesville, VA). Anti-rabbit and anti-mouse IgG complexed to horseradish peroxidase were from Amersham, Inc. (Arlington Heights, IL) or Santa Cruz. SuperSignal Chemiluminescent Substrate was obtained from Pierce (Rockford, IL).

Subjects

Muscle biopsy samples were obtained from ten (8 males, 2 females) non-diabetic subjects, 49 ± 3 years of age. The subjects were, on average, obese ($\text{BMI}=30.8 \pm 2.1 \text{ kg/m}^2$, range 23-42), with normal fasting plasma glucose ($5.4 \pm 0.2 \text{ mM}$) and HbA1c ($5.4 \pm 0.4\%$) levels. All but one of the subjects displayed normal glucose tolerance in response to a standard 75 gm OGTT; the other met the diagnostic criteria for impaired glucose tolerance. None of the subjects had a family history of type 2 diabetes. The Committee on Human Investigation of the University of California, San Diego, approved the experimental protocol. Informed written consent was obtained from all subjects after explanation of the protocol.

Cell Culture and Transfection

Skeletal muscle cell cultures were established from muscle tissue obtained by needle biopsy samples of the vastus lateralis. The method of culturing skeletal muscle cells from biopsy samples has been described in detail previously (Henry et al., 1995). Satellite cells were obtained by tryptic digestion of muscle biopsy material. Cells were propagated in culture by modifications (Henry et al., 1995) of the methods described by Blau and Webster (Blau and Webster, 1981) and Sarabia et al. (Sarabia et al., 1990). When cells attained ~80% confluency, they were transferred to differentiation media. After 48 hours of differentiation, cells were placed in OptiMEM and treated with lipid transfection reagent and specific or non-specific siRNA (final concentration = 100 nM) for 4 hours, following manufacturer's instructions, washed and then cultured in differentiation media an additional 48 hrs before protein extraction or assay of glucose uptake.

Protein Extraction

After treatment, cells were washed in serum free alpha-MEM containing 0.1% BSA and incubated for 15' at 37°C in the presence or absence of insulin (33nM). Following this treatment, separate samples of cells in 6 well plates were rapidly washed with 4°C PBS and then lysed in extraction buffer as described in detail previously (Ciaraldi et al., 1995). Cells were scraped from the plates and solubilized over 30 minutes at 4°C with vortexing. Nonsolubilized material was removed by centrifugation at 14,000×g (10 min, 4°C). Protein concentration was determined according to the method of Bradford (Bradford, 1976) and cell extracts were stored at -70°C until further analysis.

For glycogen synthase (GS) assay, cells from 6-well plates were first incubated in the presence or absence of insulin (33nM) for 30 minutes at 37°C, washed with PBS and scraped into 0.3ml of GS extraction buffer (Henry et al., 1996). Extracts were stored at -70°C before assay.

Glucose uptake assay

At the same time as protein extraction, glucose uptake was measured in parallel plates using a previously described protocol (Ciaraldi et al., 1995). Briefly, cells were washed in serum-free media, and incubated $\pm$ insulin (33nM) for 90 min at 37°C in a 5% CO₂ incubator. Uptake of the non-metabolized analog 2-deoxyglucose (final concentration=0.01 mM) was measured in triplicate over 10 minutes at room temperature (Ciaraldi et al., 1995). An aliquot of the suspension was removed for protein analysis. The uptake of L-glucose is used to correct each sample for the contribution of diffusion.

Glycogen synthase activity assay

GS activity was measured as described in detail previously (21). GS activity was determined in duplicate in diluted supernatants at a physiologic concentration of substrate (0.3 mM UDP-glucose) and expressed as nmoles of UDP-glucose incorporated into glycogen per minute per

milligram of total protein or as fractional velocity (FV = the ratio of activity assayed at 0.1 mM glucose-6-phosphate /activity at 10 mM glucose-6-phosphate).

Electrophoresis and Western Blotting

Protein extracts were size fractionated on 7.5% or 10% polyacrylamide gels according to standard procedures (Burnette, 1981). Proteins were transferred to nitrocellulose, blocked, and incubated with antibody to the particular protein of interest. Bands were visualized using secondary antibody tagged with horseradish peroxidase followed by enhanced chemiluminescence reaction. For analysis of GSK-3 and Akt phosphorylation, membranes were first probed with an antibody against the specific phosphorylated protein, then stripped and reprobed for total protein. Quantification of band intensity was performed using an Alpha Innotech MultiImage Light Cabinet and ChemiImager4000 v.4.04 software (San Leandro, CA). Results were obtained in integrated density value units per 10 μ g total protein. Final values were expressed as percentage of non-specific control for each individual set of cells.

Statistical analysis

Statistical significance was evaluated with a paired student t-test and/or Wilcoxon Signed Rank Test using GraphPad Prism V.4.0 (San Diego, CA). Significance was accepted at the $P < 0.05$ level. All data and calculated results are expressed as the mean $\pm$ standard error of the mean (SEM). Results are expressed as absolute values or normalized against the appropriate non-specific (transfection reagent + ns-siRNA) control treatment. For a given manipulation, muscle cells from each subject served as its own control. Due to limitations in cell availability not all determinations were performed for each individual. The number of independent determinations is indicated in legends to the figures and tables.

Results

GSK-3 expression

Transfection of cultured muscle cells with siRNA against GSK-3 β resulted in an approximate 60-70% reduction in GSK3 β protein expression (Figure 1). In cells from these subjects GSK-3 β represented $54 \pm 7\%$ of total GSK-3 protein. GSK-3 α expression was unaltered by this treatment, serving as an internal control for the specificity of the manipulation of gene expression. Inactivating phosphorylation of GSK-3 occurs on S21 (GSK-3 α) and S9 (GSK-3 β). Phosphorylation on these sites serve as a surrogate for enzyme activity; lower activity in the presence of increased phosphorylation (Shaw et al., 1997). While there was a weak tendency for GSK-3 β knockdown to result in increased phosphorylation of GSK-3 α in the basal ($p = 0.23$) state, the effect did not attain statistical significance. GSK-3 β knockdown also did not influence GSK-3 β phosphorylation ($p = 0.87$ and 0.69 for basal and insulin-stimulated, respectively) (Figure 2).

Regulation of Glycogen Synthase

The consequences of down regulating GSK-3 β expression on GS were evaluated at the level of activity. Activity measured in the presence of a maximal concentration of the allosteric regulator glucose-6-phosphate (G-6-P) is independent of the phosphorylation state of GS and can serve as a surrogate for the total amount of enzyme (Lawrence and Roach, 1997). Reducing GSK-3 β expression had no effect on total GS activity in muscle cells (4.04 ± 1.26 vs 4.04 ± 1.41 nmol/min/mg protein, non-specific control and GSK-3 β knockdown, respectively). This would be consistent with measures of GS protein expression ($90 \pm 17\%$ of non-specific, $n = 5$). GS fractional velocity (FV) defines the activity measured at a low [G-6-P], where control resides at the level of GS phosphorylation, with reductions in phosphorylation resulting in augmented activity (Lawrence and Roach, 1997). Down-regulation of GSK-3 β led to increases

in GS FV in the absence of insulin ($p < 0.025$) (Fig. 3). Reducing GSK-3 β expression also improved insulin action, as the absolute insulin-stimulated incremental increase in GS FV was effectively doubled (Figure 3, $p < 0.05$), compared to the paired non-specific control.

Regulation of Glucose Uptake

Unlike the behavior of GS, reducing GSK-3 β expression had no statistically significant effects on basal glucose uptake in muscle cells (Fig. 4). In control cells acute insulin exposure resulted in a modest, but statistically significant ($p < 0.05$) stimulation of uptake. Unlike the case with GSK-3 α (Ciaraldi, et al., 2007), GSK-3 β knockdown did not appreciably alter absolute insulin-stimulated activity (Fig. 4). However, the absolute insulin response, determined as the individual increment in uptake over basal with acute insulin treatment, was more than doubled when GSK-3 β expression was reduced (Figure 4).

GSK-3 β and Insulin Signaling

We have previously reported that both chemical inhibition of GSK-3 (Nikoulina et al, 2002) and GSK-3 α knockdown (Ciaraldi et al., 2007) resulted in improved insulin action associated with upregulation of IRS-1 protein expression. Possible sources of the improvements in insulin action seen with GSK-3 β knockdown were evaluated at the level of signaling protein expression and phosphorylation. Expression of IRS-1 was unaltered with GSK-3 β knockdown (Figure 5). Expression of Akt 1/2 was also unchanged with GSK-3 β knockdown (Figure 5). Phosphorylation of Akt 1/2 on S473 and T308 were followed as markers of insulin signaling. The extent of Akt phosphorylation on either S473 or T308 in the basal state did not differ between control and GSK-3 β knockdown cells (Figure 6). Insulin stimulation of Akt phosphorylation, measured at the same maximal hormone concentration where GSK-3 β knockdown improved insulin action (Fig. 3 & 4), was not significantly altered (Fig. 6).

Discussion

In the current report we present evidence that in human skeletal muscle the β -isoform of GSK-3 is involved in the regulation of GS activity in both the absence and presence of insulin in ways that differ from the involvement of GSK-3 α . GSK-3 is a broadly expressed serine kinase with a multitude of roles including determination of cell differentiation, growth, apoptosis, and regulation of protein synthesis and degradation (Doble and Woodgett, 2003). These actions are in addition to its initial discovery as the primary kinase controlling glycogen synthase activity (Embi et al., 1980). Attention has been paid to a potential role for GSK-3 in the development of insulin resistance. Several lines of evidence support such a role. 1) Elevated expression and activity of GSK-3 has been found in skeletal muscle of poorly controlled type 2 diabetics (Nikoulina et al., 2000) and adipose tissue of insulin resistant rats (Eldar-Finkelman et al., 1999), though it should be noted that others have found GSK-3 expression and activity to be normal in skeletal muscle from well controlled diabetics (Hojlund et al., 2003). 2) Interventions that improve action such as thiazolidinedione treatment (Ciaraldi et al., 2006), acute exercise (Sakamoto et al., 2004; Wojtazewski et al., 2001), and chronic exercise combined with weight loss (Venojarvi et al., 2004) reduce GSK-3 expression and/or activity in human skeletal muscle or adipose tissue. 3) A number of chemically distinct and selective GSK-3 inhibitors have been shown to improve insulin action in cultured cells, in isolated muscle and in vivo in animal models of insulin resistance (reviewed in (Wagman et al., 2004)). While much of the most convincing evidence for an involvement of GSK-3 in insulin resistance has come from inhibitor studies, such agents are not able to discriminate between isoforms.

GSK-3 β has been the most studied isoform, due in part to the initial availability of specific reagents (Doble and Woodgett, 2003). The isoforms differ in ways other than just their

structures. While GSK-3 α and - β are both present in the cytosol, - β is also found in nuclei and mitochondria, unlike GSK-3 α (Bijur and Jope, 2003 ; Hoshi et al., 1995). Subcellular localization of GSK-3 can play an important role in regulation of enzyme activity, as stimulation of apoptotic signaling in neuronal cells activates the nuclear and mitochondrial pools of GSK-3 β , without influencing cytosolic GSK-3 β (Bijur and Jope, 2003). Distribution of GSK-3 throughout the cells into distinct pools, localized with specific substrates, could also help explain why certain external stimuli can influence GSK-3 activity toward β -catenin and not GS; the converse is also true (Ding et al., 2000). Two groups working in humans reported exercise-induced changes in muscle GSK-3 α activity with no influence on - β (Sakamoto et al., 2004; Wojtazewski et al., 2001). GSK-3 α activity in human skeletal muscle cells (Gaster et al., 2002) and muscle tissue (Hojlund et al., 2003; Nikoulina et al., 2000) was also found to be more responsive to acute insulin treatment than - β . These results, combined with the stronger association between GSK-3 α and insulin-stimulated whole body glucose disposal rate, and muscle GS activity (Nikoulina et al., 2000), led us to hypothesize that GSK-3 α may mediate much of the insulin effect on glycogen formation in muscle, at least in humans. GSK-3 β could potentially be playing more of a role in basal regulation of metabolic activities. This last supposition would be consistent with the observation that the strongest correlation to GSK-3 β in human muscle was with basal (fasting) GS activity (Nikoulina et al., 2000). However, expression of a mutant GSK-3 β that could not be inhibited by phosphorylation partially reduced the GS response (Summers et al., 1999) to insulin. In addition, muscle-specific overexpression of GSK-3 β resulted in glucose intolerance in the presence of increased fasting insulin levels, though only in male mice (Pearce et al., 2004). While information is available about differential roles of GSK-3 α and - β in control of neuronal survival (Liang and Chuang, 2007), cardiogenesis (Lee et al., 2007) and transcription (Liang and Chuang, 2006), less is known about GSK-3 isoform specificity in metabolic regulation. The most detailed evaluation of this issue was performed by McManus et al. using homozygous mice where constitutively activated GSK-3 α and/or - β were knocked in (McManus et al., 2005). They concluded that GSK-3 β was the major isoform involved in insulin activation of glycogen synthase in skeletal muscle. Recently, MacAulay et al, using GSK-3 α knockout mice, found major effects on hepatic glycogen metabolism and postulated the existence of tissue-specific functions of GSK-3 isoforms (MacAulay et al., 2007). These findings conflict somewhat with our results, which indicate that both GSK-3 α (Ciaraldi et al., 2007) and - β can participate in insulin action in muscle. Species differences could contribute in large part to the varying results as, while McManus et al reported that GSK-3 α in mouse skeletal muscle was not influenced by insulin (McManus et al., 2005), there are multiple reports in human muscle of GSK-3 α being equally or more responsive than - β to insulin (Borthwick et al., 1995; Gaster et al., 2004; Hojlund et al., 2003; Nikoulina et al., 2000). A further difference between species is that in human muscle tissue and cells GSK-3 β is usually only modestly (50-60% of total GSK-3 protein) more abundant than - α (Borthwick et al., 1995; Gaster et al., 2004; Venojarvi et al., 2004) , while in mouse muscle GSK-3 β protein expression is 4-fold higher than that of - α (McManus et al., 2005).

We tested the question of isoform differences regarding insulin action in human muscle directly by reducing GSK-3 β expression with specific siRNA. This approach was taken to permit comparison to the global reduction in GSK-3 activity and expression attained by treatment with a selective GSK-3 inhibitor (Nikoulina et al., 2002). Comparing the effects of global chemical inhibition of GSK-3 (Nikoulina et al., 2002) with isoform-specific knockdown ((Ciaraldi et al., 2007) and the present work) should help determine if - α and - β do play different roles in the regulation of metabolism in human muscle. In making any such comparisons it is important to keep in mind the potential quantitative effects of the different manipulations on GSK-3 activity. As GSK-3 α and - β are expressed at near similar levels in human skeletal muscle tissue (Lau et al, 1999; Nikoulina et al., 2000; Venojarvi et al., 2004) and muscle cells (Borthwick et al., 1995; Gaster et al., 2004; Nikoulina et al., 2002), knockdown of 60-70% of

one isoform would be expected to reduce total activity by 30-40%, while the specific inhibitor we employed reduced enzyme activity by ~90% (Nikoulina et al., 2002). Several differences are apparent between these experimental approaches to manipulating GSK-3. While chronic treatment with selective inhibitors increased basal glucose uptake in human muscle cells (Nikoulina et al., 2002), knockdown of GSK-3 α (Ciaraldi et al., 2007) had no such effect, while β knockdown was intermediate, suggesting that basal uptake may not be under the physiologic regulation of GSK-3 α , or possibly β . That would be consistent with the report that chemical inhibition of GSK-3 had no acute effect on basal glucose uptake in skeletal muscle of normal or diabetic rats (Ring et al., 2003). The ability of GSK-3 inhibition to increase basal GS activity was shared with GSK-3 β knockdown, while GSK-3 α had no such effect (Ciaraldi et al., 2007). This would indicate that basal GS activity could be under the control of GSK-3 β . While no further insulin stimulation of GS was seen following inhibitor treatment, due to the large increase in basal FV, all three treatments improved insulin responsiveness for GU, suggesting that both isoforms can influence insulin action in human muscle.

The mechanisms by which GSK-3 regulates insulin action may differ between isoforms, as both GSK-3 α knock down (Ciaraldi et al., 2007) and chemical inhibition (Nikoulina et al., 2002) led to increases in IRS-1 protein expression, while GSK-3 β knockdown did not, suggesting that GSK-3 α is responsible for this effect. GSK-3 has been shown to directly phosphorylate IRS-1, impeding its ability to mediate insulin signaling (Eldar-Finkelman and Krebs, 1999; Liberman and Eldar-Finkelman, 2005). Augmented serine phosphorylation of IRS-1 can also increase degradation of the protein (Smith et al., 1996) and it is possible that GSK-3 α is phosphorylating and targeting a specific pool of IRS-1 toward the degradative pathway. However, GSK-3 β does not appear to be involved in regulating IRS-1 expression, another difference between the forms.

Cultured human skeletal muscle cells have proven to be a useful system to study the metabolic perturbations present in insulin resistant states. Multiple investigators have shown that muscle cells from type 2 diabetic subjects retain many aspects of the altered metabolic phenotype seen in vivo. These include reduced basal glucose uptake (Ciaraldi et al., 1995) and glycogen synthase (Gaster et al., 2002; Henry et al., 1996) activities, as well as insulin resistance for stimulation of these two processes. All of these defects in diabetic muscle cells are ameliorated by treatment with selective GSK-3 inhibitors (Nikoulina et al., 2002). However, only certain aspects of the diabetic metabolic phenotype are reversed by isoform-specific knockdown of GSK-3. As the isoform specific knockdown was titrated to model the diabetes-related differences seen in vivo, it seems that variations in GSK-3 expression or activity may not contribute to the reduced basal glucose uptake seen in diabetic muscle. The situation is more complicated for basal GS activity. Here it does seem that an increase in GSK-3 β , at least relative to GS, could contribute to the lower muscle GS activity seen in the fasting state in diabetes (Henry et al., 1996; Thornburn et al., 1991). The same was not true for GSK-3 α . The relative expression of GSK-3 and GS may be an important factor, as a reciprocal relationship has been shown between GSK-3 and GS expression in cultured muscle cells (MacAulay et al., 2005). What the results indicate is that relative changes in GSK-3 are only one potential contributor to the diabetic metabolic phenotype.

In summary, multiple experimental approaches have identified GSK-3 as an important regulator of both insulin action and glucose metabolism. While both GSK-3 α and β influence insulin signaling, they may do so through different mechanisms; GSK-3 β also has an additional role to reduce GS activity in the absence of insulin. The relative abundance and roles of the GSK-3 α and β isoforms also differ between species, highlighting the need for further investigation in human systems. Therapeutic targeting of either or both isoforms has the potential to provide considerable benefit for the control of glucose intolerance and insulin resistance.

Acknowledgments

This work was supported by grant RO1-DK-258291 from the National Institutes of Health (RRH), grants from the Medical Research Service, Department of Veterans Affairs and VA San Diego Healthcare System, the American Diabetes Association (RRH, TPC), and grant M01 RR-00827 in support of the General Clinical Research Center from the General Clinical Research Branch, Division of Research Sources, NIH.

References

- Bijur G, Jope RS. Glycogen synthase kinase-3 β is highly activated in nuclei and mitochondria. *Neurochemistry* 2003;14:2415–2419.
- Blau HM, Webster C. Isolation and characterization of human muscle cells. *Proc. Natl. Acad. Sci. USA* 1981;78:5623–5627. [PubMed: 6946499]
- Borthwick AC, Wells AM, Rochford JJ, Hurel SJ, Turnbull DM, Yeaman SJ. Inhibition of glycogen synthase kinase-3 by insulin in cultured human skeletal muscle myoblasts. *Biochem. Biophys. Res. Comm* 1995;210:738–745. [PubMed: 7763247]
- Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem* 1976;71:248–254. [PubMed: 942051]
- Burnette WN. “Western blotting” electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem* 1981;112:195–203. [PubMed: 6266278]
- Ciaraldi TP, Abrams L, Nikoulina S, Mudaliar S, Henry RR. Glucose transport in cultured human skeletal muscle cells. Regulation by insulin and glucose in nondiabetic and non-insulin-dependent diabetes mellitus subjects. *J. Clin. Invest* 1995;96:2820–282. [PubMed: 8675652]
- Ciaraldi TP, Oh DK, Christiansen L, Nikoulina SE, Kong APS, Baxi S, Mudaliar S, Henry RR. Tissue-specific expression and regulation of glycogen synthase kinase-3 (GSK-3) in human skeletal muscle and adipose tissue. *Am. J. Physiol. Endocrinol. Metab* 2006;291:E891–E898. [PubMed: 16757548]
- Ciaraldi TP, Nikoulina SE, Bandukwala RA, Carter L, Henry RR. Role of GSK3 α in insulin action in cultured human skeletal muscle cells. *Endocrinology* 2007;148:4393–4399. [PubMed: 17569761]
- Cline GW, Johnson K, Regittnig W, Perret P, Tozzo E, Xiao L, Damico C, Shulman GI. Effects of a novel glycogen synthase kinase-3 inhibitor on insulin-stimulated glucose metabolism in Zucker diabetic fatty (fa/fa) rats. *Diabetes* 2002;51:2903–2910. [PubMed: 12351425]
- Coghlan MP, Culbert AA, Cross DAE, Corcoran SL, Yates JW, Pearce NJ, Rausch OL, Murphy GJ, Carter PS, Roxbee Cox L, Mills D, Browen MJ, Haigh D, Ward RW, Smith DG, Murray KJ, Reith AD, Holder JC. Selective small molecule inhibitors of glycogen synthase kinase-3 modulate glycogen metabolism and gene transcription. *Chem. & Biol* 2000;7:793–803. [PubMed: 11033082]
- Cohen P, Goedert M. GSK3 inhibitors: development and therapeutic potential. *Nature Rev. Drug Discovery* 2004;3:479–487.
- Ding VW, Chen RH, McCormick F. Differential regulation of glycogen synthase kinase-3 β by insulin and wnt signaling. *J. Biol. Chem* 2000;275:32475–32481. [PubMed: 10913153]
- Doble BW, Woodgett JR. GSK-3: tricks of the trade for a multi-tasking kinase. *J. Cell. Sci* 2003;116:1175–1186. [PubMed: 12615961]
- Dokken BB, Henriksen EJ. Chronic selective glycogen synthase kinase-3 inhibition enhances glucose disposal and muscle insulin action in pre-diabetic obese Zucker rats. *Am. J. Physiol. Endocrinol. Metab* 2006;291:E207–E213. [PubMed: 16478771]
- Eldar-Finkelmann H, Krebs EG. Phosphorylation of insulin receptor substrate 1 by glycogen synthase kinase 3 impairs insulin action. *Proc. Natl. Acad. Sci. USA* 1997;94:9660–9664. [PubMed: 9275179]
- Eldar-Finkelmann H, Schreyer S, Shinohara M, LeBoeuf R, Krebs E. Increased glycogen synthase kinase-3 activity in diabetes- and obesity-prone C57BL/6J mice. *Diabetes* 1999;48:1662–1666. [PubMed: 10426388]
- Embi N, Rylatt DB, Cohen P. Glycogen synthase kinase-3 from rabbit skeletal muscle. Separation from cyclic-AMP-dependent protein kinase and phosphorylase kinase. *Eur. J. Biochem* 1980;107:519–527. [PubMed: 6249596]

- Gaster M, Petersen I, Hojlund K, Poulsen P, Beck-Nielsen H. The diabetic phenotype is conserved in myotubes established from diabetic subjects: evidence for primary defects in glucose transport and glycogen synthase activity. *Diabetes* 2002;51:921–927. [PubMed: 11916908]
- Gaster M, Brusgaard K, Handberg A, Hojlund K, Wojtaszewski JFP, Beck-Nielsen H. The primary defect in glycogen synthase activity is not based on increased glycogen synthase kinase-3 α activity in diabetic myotubes. *Biochem. Biophys. Res. Comm* 2004;319:1235–1240. [PubMed: 15194499]
- Hansen L, Arden KC, Rasmussen SB, Viars CS, Vestergaard H, Hansen T, Moller AM, Woodgett JR, Pedersen O. Chromosomal mapping and mutational analysis of the coding region of the glycogen synthase kinase-3 α and β isoforms in patients with NIDDM. *Diabetologia* 1997;40:940–946. [PubMed: 9267989]
- Henry RR, Abrams L, Nikoulina S, Ciaraldi T. Insulin action and glucose metabolism in nondiabetic control and NIDDM subjects. Comparison using human skeletal muscle cell cultures. *Diabetes* 1995;44:936–946. [PubMed: 7622000]
- Henry RR, Ciaraldi TP, Abrams-Carter L, Mudaliar S, Park KS, Nikoulina SE. Glycogen synthase activity is reduced in cultured skeletal muscle cells of non-insulin-dependent diabetes mellitus subjects. *J. Clin. Invest* 1996;98:1231–1236. [PubMed: 8787686]
- Hoeflich KP, Luo J, Rubie EA, Tsao M-S, Jin O, Woodgett JR. Requirement for glycogen synthase kinase 3- β in cell survival and NF-kappaB activation. *Nature* 2000;406:86–90. [PubMed: 10894547]
- Hojlund K, Staehr P, Falck Hansen B, Green KA, Hardie DG, Richter EA, Beck-Nielsen H, Wojtaszewski JFP. Increased phosphorylation of skeletal muscle glycogen synthase at NH₂-terminal sites during physiological hyperinsulinemia in type 2 diabetes. *Diabetes* 2003;52:1393–1402. [PubMed: 12765949]
- Hoshi M, Sato M, Kondo S, Takashima A, Noguchi K, Takahashi M, Ishiguro K, Imahori K. Different localization of tau protein kinase I/Glycogen synthase kinase-3 β from glycogen synthase kinase-3 α in cerebellum mitochondria. *J. Biochem* 1995;118:683–685. [PubMed: 8576078]
- Lau KF, Miller CC, Anderton BH, Shaw PC. Expression analysis of glycogen synthase kinase-3 in human tissues. *J. Peptide Res* 1999;54:85–91. [PubMed: 10448973]
- Lawrence JC, Roach PJ. New insights into the role and mechanism of glycogen synthase activation by insulin. *Diabetes* 1997;46:541–547. [PubMed: 9075792]
- Lee H-C, Tsai J-N, Liao P-Y, Tsai W-Y, Lin K-Y, Chuang C-C, Sun C-K, Chang W-C, Tsai H-J. Glycogen synthase kinase 3 α and 3 β have distinct functions during cardiogenesis of zebrafish embryo. *BMC Devel. Biol* 2007;7:93–107. [PubMed: 17683539]
- Liang M-H, Chuang D-M. Differential roles of glycogen synthase kinase-3 isoforms in the regulation of transcriptional activation. *J. Biol. Chem* 2006;281:30479–30484. [PubMed: 16912034]
- Liang M-H, Chuang D-M. Regulation and function of glycogen synthase kinase-3 isoforms in neuronal survival. *J. Biol. Chem* 282:3904–3917. [PubMed: 17148450]
- Lieberman Z, Eldar-Finkelman H. Serine 332 phosphorylation of insulin receptor substrate-1 by glycogen synthase kinase-3 attenuates insulin signaling. *J. Biol. Chem* 2005;280:4422–4428. [PubMed: 15574412]
- MacAulay K, Blair AS, Hajdich E, Terashima T, Baba O, Sutherland C, Hundal HS. Constitutive activation of GSK3 down-regulates glycogen synthase abundance and glycogen deposition in rat skeletal muscle cells. *J. Biol. Chem* 2005;280:9509–9518. [PubMed: 15632169]
- MacAulay K, Doble BW, Patel S, Hansoita T, Sinclair EM, Drucker DJ, Nagy A, Woodgett JR. Glycogen synthase kinase 3 α -specific regulation of murine hepatic glycogen metabolism. *Cell Metabolism* 2007;6:329–337. [PubMed: 17908561]
- McManus E, Sakamoto K, Armit LJ, Ronaldson L, Shapiro N, Marquez R, Alessi DR. Role that phosphorylation by GSK3 plays in insulin and Wnt signalling defined by knockin analysis. *EMBO J* 2005;24:1571–1583. [PubMed: 15791206]
- Nikoulina SE, Ciaraldi TP, Mudaliar S, Mohideen P, Carter L, Henry RR. Potential role of glycogen synthase kinase 3 in skeletal muscle insulin resistance of Type 2 diabetes. *Diabetes* 2000;49:263–271. [PubMed: 10868943]
- Nikoulina SE, Ciaraldi TP, Mudaliar S, Carter L, Johnson K, Henry RR. Inhibition of glycogen synthase kinase 3 improves insulin action and glucose metabolism in human skeletal muscle. *Diabetes* 2002;51:2190–2198. [PubMed: 12086949]

- Pearce NJ, Arch JRS, Clapham JC, Coghlan MP, Corcoran SL, Lister CA, Llano A, Moore GB, Murphy GJ, Smith SA, Taylor CM, Yates JW, Morrison AD, Harper AJ, Roxbee-Cox L, Abuin A, Wargent E, Holder JC. Development of glucose intolerance in male transgenic mice overexpressing human glycogen synthase kinase-3 β on a muscle-specific promoter. *Metabolism* 2004;53:1322–1333. [PubMed: 15375789]
- Ring DB, Johnson KW, Henriksen EJ, Nuss JM, Goff D, Kinnick TR, Ma ST, Reeder JW, Samuels I, Slabik T, Wagman AS, Wernette Hammond M-E, Harrison SD. Selective glycogen synthase kinase 3 inhibitors potentiate insulin activation of glucose transport and utilization in vitro and in vivo. *Diabetes* 2003;52:588–595. [PubMed: 12606497]
- Sakamoto K, Arnolds DEW, Ekberg I, Thorell A, Goodyear LJ. Exercise regulates Akt and glycogen synthase kinase-3 activities in human skeletal muscle. *Biochem. Biophys. Res. Comm* 2004;319:419–425. [PubMed: 15178423]
- Sarabia V, Ramlal T, Klip A. Glucose uptake in human and animal muscle cells in culture. *Biochem. Cell Biol* 1990;68:536–542. [PubMed: 2188683]
- Shaw M, Cohen P, Alessi DR. Further evidence that the inhibition of glycogen synthase kinase-3 β by IGF-1 is mediated by PDK1/PKB-induced phosphorylation of Ser-9 and not by dephosphorylation of Tyr-216. *FEBS Letts* 1997;416:307–311. [PubMed: 9373175]
- Smith LK, Rice KM, Garner CW. The insulin-induced down-regulation of IRS-1 in 3T3-L1 adipocytes is mediated by a calcium-dependent thiol protease. *Mol. Cell. Endocrinol* 1996;122:81–92. [PubMed: 8898350]
- Summers SA, Kao AW, Kohn AD, Backus GS, Roth RA, Pessin JE, Birnbaum MJ. The role of glycogen synthase kinase 3 β in insulin-stimulated glucose metabolism. *J. Biol. Chem* 1999;274:17934–17940. [PubMed: 10364240]
- Thornburn AW, Gumbiner B, Bulacan F, Brechtel G, Henry RR. Multiple defects in muscle glycogen synthase activity contribute to reduced glycogen synthesis in non-insulin dependent diabetes mellitus. *J. Clin. Invest* 1991;87:489–495. [PubMed: 1899428]
- Venojarvi M, Puhke R, Hamalainen H, Marniemi J, Rastas M, Rusko H, Nuutila P, Hanninen O, Aunola S. Role of skeletal muscle-fibre type in regulation of glucose metabolism in middle-aged subjects with impaired glucose tolerance during a long-term exercise and dietary intervention. *Diab. Ob. Metab* 2004;7:745–754.
- Wagman AS, Johnson KW, Bussiere DE. Discovery and development of GSK3 inhibitors for the treatment of type 2 diabetes. *Curr. Pharmaceut. Design* 2004;10:1105–1137.
- Wojtazewski JFP, Nielsen P, Kiens B, Richter EA. Regulation of glycogen synthase kinase-3 in human skeletal muscle. effects of food intake and bicycle exercise. *Diabetes* 2001;50:265–269. [PubMed: 11272135]
- Woodgett JR. Physiological roles of glycogen synthase kinase-3: Potential as a therapeutic target for diabetes and other disorders. *Curr. Drug Targets Immune Endocr. Metabol. Disord* 2003;3:281–290. [PubMed: 14683459]

Figure 1.

Knockdown of GSK-3 β in human skeletal muscle cells. **A)** Representative western blot for total GSK-3 in cells treated with non-specific (cont-control) or specific siRNA, as described in the Methods. **B)** Quantification of western blots for GSK-3 α and GSK-3 β protein expression. Results expressed as % of non-specific siRNA (control) treated cells for each individual. Results depicted as mean + SEM, n=10.

*p<0.001 vs. non-specific/control siRNA

Figure 2.

Effect of GSK-3 β knockdown on GSK-3 phosphorylation. Cell proteins extracted after 15 min incubation in the absence (basal) or presence (+ Ins) of insulin (33 nM); inc = absolute insulin increment (+Ins – basal). pS²¹-GSK-3 α and pS⁹-GSK-3 β determined by western blotting. Blots were stripped and reprobed for total GSK-3. Results normalized against the amount of GSK-3 for each condition, average + SEM. Open bars – control, solid bars – GSK-3 β knockdown, n=9.

*p<0.05 vs paired basal

Figure 3. Effects of GSK-3 β knockdown on glycogen synthase activity

GS activity measured in cells extracted after 30 min incubation in the absence (basal) or presence of 33 nM insulin in control (open bars) and GSK-3 β knockdown (solid bars) cells. Results expressed as the GS FV or absolute insulin increment (inc) in GS FV for cells from each subject. Results are average $\pm$ SEM, n=9.

* p<0.05 vs paired basal

† p<0.05 vs. control siRNA.

Figure 4. Effects of GSK-3 β knockdown on glucose uptake activity

GS activity measured in cells extracted after 90 min incubation in the absence (basal) or presence of 33 nM insulin in control (open bars) and GSK-3 β knockdown (solid bars) cells. Results expressed as the initial rate of glucose uptake or absolute insulin increment (+Ins – basal) for cells from each subject, Results are average + SEM, n=8.

* p<0.05 vs paired basal

† p<0.05 vs. control siRNA.

Figure 5. Insulin signaling protein expression after downregulation of GSK-3 β . A) Representative western blots for IRS-1 and Akt 1/2. B) Quantification of blots. Results presented as percent of expression in control (non-specific) cells for each individual. Results are mean + SEM. For IRS-1 n=8, for Akt 1/2, n=9.

Figure 6.

Effect of downregulation of GSK-3 β on Akt phosphorylation. Cell proteins extracted after 15 min incubation in the absence (basal) or presence of insulin (33 nM). pS⁴⁷³- or pT³⁰⁸-Akt determined by western blotting. Blots were stripped and reprobed for Akt-1/2. Quantitation of blots: A) pS⁴⁷³-Akt. B) pT³⁰⁸-Akt. Results normalized against the amount of Akt for each condition, average + SEM, inc = absolute insulin increment. Open bars – control, solid bars – GSK-3 β knockdown, n=9.

*p<0.05 vs basal