Development of Glucose Intolerance in Male Transgenic Mice Overexpressing Human Glycogen Synthase Kinase-3β on a Muscle-Specific Promoter

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Glycogen synthase kinase-3 (GSK-3) protein levels and activity are elevated in skeletal muscle in type 2 diabetes, and inversely correlated with both glycogen synthase activity and insulin-stimulated glucose disposal. To explore this relationship, we have produced transgenic mice that overexpress human GSK-3β in skeletal muscle. GSK-3β transgenic mice were heavier, by up to 20% ($P < .001$), than their age-matched controls due to an increase in fat mass. The male GSK-3β transgenic mice had significantly raised plasma insulin levels and by 24 weeks of age became glucose-intolerant as determined by a 50% increase in the area under their oral glucose tolerance curve ($P < .001$). They were also hyperlipidemic with significantly raised serum cholesterol (+90%), nonesterified fatty acids (NEFAs) (+55%), and triglycerides (+170%). At 29 weeks of age, GSK-3β protein levels were 5-fold higher, and glycogen synthase activation (−27%), glycogen levels (−58%) and insulin receptor substrate-1 (IRS-1) protein levels (−67%) were significantly reduced in skeletal muscle. Hepatic glycogen levels were significantly increased 4-fold. Female GSK-3β transgenic mice did not develop glucose intolerance despite 7-fold overexpression of GSK-3β protein and a 20% reduction in glycogen synthase activation in skeletal muscle. However, plasma NEFAs and muscle IRS-1 protein levels were unchanged in females. We conclude that overexpression of human GSK-3β in skeletal muscle of male mice resulted in impaired glucose tolerance despite raised insulin levels, consistent with the possibility that elevated levels of GSK-3 in type 2 diabetes are partly responsible for insulin resistance.
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GLYCOGEN SYNTHESIS in skeletal muscle accounts for a large proportion of insulin-stimulated glucose disposal in the whole body. Its suppression is a major contributor to reduced whole body glucose disposal in impaired glucose tolerance and type 2 diabetes.[1,2] The final step in the pathway of glycogen synthesis is catalyzed by glycogen synthase. The activity of glycogen synthase is inhibited by phosphorylation by glycogen synthase kinase-3 (GSK-3) and stimulated by dephosphorylation mediated by protein phosphatase-1G (PP1G).[3]

The activity of GSK-3 is elevated and inversely correlated with glycogen synthase activity in skeletal muscle of type 2 diabetic subjects.[4] However, whether this contributes to insulin resistance in type 2 diabetic subjects is unclear. ^{13}C and ^{31}P nuclear magnetic resonance (NMR) studies indicate that the majority of the control of glycogen synthesis in skeletal muscle is exerted at the glucose transport, rather than at glycogen synthase or the intermediary hexokinase step.[5] Thus, the increase in glucose-6-phosphate concentration during a hyperinsulinemic euglycemic clamp was less in type 2 diabetic than in control subjects. Activation of glycogen synthase by insulin has been suggested to serve merely to prevent the concentration of glucose-6-phosphate from rising by too much when glucose transport is stimulated.[6]

Some workers have questioned whether the concentration of glucose-6-phosphate can be accurately determined by NMR studies and whether crossover analysis of the estimated changes in glucose-6-phosphate concentration may be oversimplistic.[7] Turning to other approaches, there is evidence that in monkeys activation of glycogen synthesis by insulin is associated with a decrease in the glucose-6-phosphate content of muscle,[8] which suggests that glucose is "pulled" into glycogen by activation of glycogen synthase, rather than being "pushed" by activation of glucose transport or hexokinase. Overexpression of hexokinase has been found to increase the rate of glycogen accumulation,[9] while overexpression of glycogen synthase markedly increased muscle glycogen content.[10] By contrast, there are conflicting reports concerning the effect of overexpressing the insulin-sensitive glucose transporter GLUT-4 in skeletal muscle.[11-13] These results and others[14] suggest that the rate of glycogen synthesis can be regulated at the transport, phosphorylation, or glycogen synthase steps.[15]

It is also important to recognize that the influence of GSK-3 on insulin action may not depend solely on the contribution of glycogen synthase to the control of glycogen synthesis. GSK-3 may inhibit insulin action not only by phosphorylating and inhibiting glycogen synthase but also by phosphorylating serine and threonine residues on insulin receptor substrate-1 (IRS-1).[16] This enhances IRS-1 degradation and reduces its interaction with the insulin receptor.[17] Phosphorylation of IRS-1 by GSK-3 would thus be expected to inhibit glucose transport. GSK-3 has a number of other targets,[18] but these are less obviously linked to insulin-stimulated glucose disposal in skeletal muscle.

GSK-3 is a serine/threonine kinase that exists as 2 isoforms, α and β. To address its role in the control of glycogen synthesis, and more generally in insulin sensitivity and glucose tolerance, we have generated a line of mice that overexpresses GSK-3β in skeletal muscle. GSK-3α–overexpressing mice will be described in a subsequent publication.

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MATERIALS AND METHODS

Generation of Transgenic Mice Overexpressing Human GSK-3β

Human GSK-3β cDNA was cloned into the vector Pact-IVSpolyA. This construct (Pact-GSK3β-IVSpolyA) contained the human alpha-actin promoter sequences that drive skeletal muscle specific expression. The transgene was linearized and separated from the plasmid vector backbone by digestion with *Not1* and *Kpn1*. The transgene was injected into the pronuclei of fertilised eggs (F2 hybrid strain [C57BL/6 × CBA]) using standard transgenic techniques.[19] Founder animals containing the transgene were identified using Southern blot analysis. From these founders a line displaying elevated levels of GSK-3β protein in skeletal muscle was identified using Western blot analysis. Heterozygous animals from this hybrid line were interbred to generate mice homozygous for the transgene Pact-GSK3β-IVSpolyA maximizing the levels of GSK-3β overexpression.

Phenotyping Studies

Male and female homozygous human GSK-3β–overexpressing transgenic mice and age-matched control C57BL/6 mice were maintained on a 12-hour light/12-hour dark cycle (lights on at 6 AM GMT) with free access to a normal diet (Teklad 2014, Madison, WI) and water. Weekly measurements of body weight were started at 6 weeks of age. Analysis of body composition by dual-energy x-ray absorptiometry (DEXA) was performed on mice of 6 and 25 weeks of age, oral glucose tolerance tests were performed on mice of 10 and 24 weeks of age, and terminal tissues were taken from mice of 29 weeks of age. Since the transgenic mice were on a mixed C57BL/6 and CBA background, whereas the controls were pure C57BL/6, a subsequent experiment was conducted comparing the 2 background strains and a mixed B6CBAF1/OlaHsd strain (Harlan-Olac, Bicester, UK). All procedures were in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986.

Preparation of Tissues

GSK-3β–overexpressing transgenic mice and C57BL/6 control mice, fasted for 6 hours, were killed by cervical dislocation. Liver, heart, skeletal muscle (mixed fiber gastrocnemius and plantaris) and perigenital white, subcutaneous white (inguinal), and interscapular brown adipose tissue were immediately removed, placed into aluminium foil, and freeze-clamped in liquid nitrogen. Tissue samples were then stored at −80°C until assayed.

mRNA Analysis

To measure the mRNA levels of the human GSK-3β transgene and the murine housekeeping genes hypoxanthine guaninine phosphoribosyl transferase (HPRT) and β_2-microglobulin, total RNA was isolated from the frozen tissue samples and reverse-transcribed as described previously.[20] Comparative mRNA-derived cDNA measurements were then performed using real-time polymerase chain reaction (PCR)-based 5′ nuclease assays (TaqMan assays) designed from GenBank nucleotide sequences using Primer Express software version 1.0 (Applied Biosystems, Warrington, UK), also as previously described.[20] Each set of real-time PCR oligonucleotides consisted of primers (Sigma-Genosys Ltd, Cambridge, UK) and a TaqMan probe labeled with the fluorescent reporter dye 6-carboxyfluorescein (FAM) at the 5′-end and the quencher dye 6-carboxytetramethylrhodamine (TAMRA) at the 3′-end (Applied Biosystems, Warrington, UK). Oligonucleotide sequences were as follows: human GSK-3β transgene mRNA: forward primer (specific to the human α-actin 5′ UTR) 5′-CCAGTCGGTTCACCTGGTCA-3′; probe (specific to the human α-actin 5′ UTR) FAM-5′-CCTCGTCTCCGGAGCCACACGC-TAMRA-3′; reverse primer (specific to the human GSK-3β coding region) 5′-AGCTCTCCGCAAAGGAGGTG-3′; mouse β_2-microglobulin mRNA: forward primer 5′-GCCTGCAGAGTTAAGCATGACAG-3′; probe FAM-5′-TCACATGTCTCGATCCCAGTAGACGGTCTT-3′-TAMRA; reverse primer 5′-AGGTTCAAATGAATCTTCAGAGCATC-3′. Sequences of the mouse HPRT mRNA oligonucleotides were as reported previously.[20] For each transcript, equal amounts of cDNAs (equivalent to 12.5 ng total RNA) were assayed together with RNA blank and PCR reagent controls and a set of standards consisting of a 3-fold serial dilution of transgenic gastrocnemius/plantaris muscle cDNA, equivalent to 150 to 0.0025 ng total RNA.

Western Blotting

Frozen tissue samples were ground to a fine powder under liquid nitrogen prior to homogenization in buffer (6 mL/g of tissue of 50 mmol/L Tris-HCl, pH 7.5, 10 mmol/L NaF, 5 mmol/L $Na_4P_2O_7$, 1 mmol/L Na_3VO_4, 10 mmol/L β-glycerophosphate, 1 mmol/L EDTA, 1 mmol/L EGTA, 0.1% β-mercaptoethanol, 0.27 mol/L sucrose and protease inhibitor cocktail ["Complete," Boehringer Mannheim, Mannheim Germany]) using an Ultra Turrax homogenizer (IKA, Janke & Kunkel Staufen, GA). Triton-X-100 was added to 0.5% (vol/vol) and the homogenates were centrifuged at 10,000 × *g* for 20 minutes at 4°C. Supernatants were removed and assayed for protein content[21] prior to storage at −80°C. Protein samples were resolved by sodium dodecyl sulfate polyacrylamide gel electropheresis (SDS-PAGE; 4% to 12% gradient gels, each loaded with 20 μg or 30 μg of total protein per lane) and transferred to polyvinylidine flouride (PVDF) membrane. Nonspecific binding sites were blocked by incubation in Tris-buffered saline (TBS)-tween containing 5% non-fat dry milk. GSK-3β was detected by incubation with a monoclonal anti–GSK-3β antibody (Transduction Laboratories, cat. no. G22320, BD Biosciences, San Jose, CA), which recognizes both mouse and human GSK-3β, and IRS-1 was detected with a polyclonal anti–IRS-1 antibody (Upstate, Milton Keynes, UK, cat. no. 06-248) followed by either an anti-mouse (GSK-3 blots) or an anti-rabbit (IRS-1 blots) horse-radish peroxidase (HRP)-conjugated secondary antibody (Amersham [Buckinghamshire, UK] or Perbio [Cheshire, UK]) and visualized using Supersignal West Dura (Pierce, Rockford, IL) chemiluminescent substrate.

Body Composition

Body composition was determined by DEXA (Lunar Piximus densitometer; Lunar Corp, Madison, WI). Prior to each session, the densitometer was calibrated using a manufacturer-supplied quality-control phantom for bone mineral density and percentage fat. Mice were anesthetized with isoflurane (1.5%) during data acquisition; scan cycles were typically completed in 4 to 5 minutes per animal. After completion of the scan, body composition (fat free mass, fat mass, and, for the right femur, bone mineral density) for the whole animal was provided by the system's most recent software, version 1.45.

Glycogen Synthase Activity Assay

Homogenates of frozen liver and muscle tissue samples were prepared as described for Western blotting, but without the addition of Triton X-100. Samples were centrifuged at 26,000 × *g* for 15 minutes at 4°C and the supernatant isolated, frozen, and stored at −80°C in aliquots prior to assay. The glycogen synthase activity of the muscle and liver supernatants was then measured, in the presence or absence of 20 mmol/L glucose 6-phosphate, as described previously.[22] Results are expressed as the ratio of glycogen synthase activity in the presence and absence of glucose 6-phosphate.

Glycogen Content

Liver and muscle glycogen contents were determined by the α-amyloglucosidase method.[23,24] The glycogen-derived glucose content of the

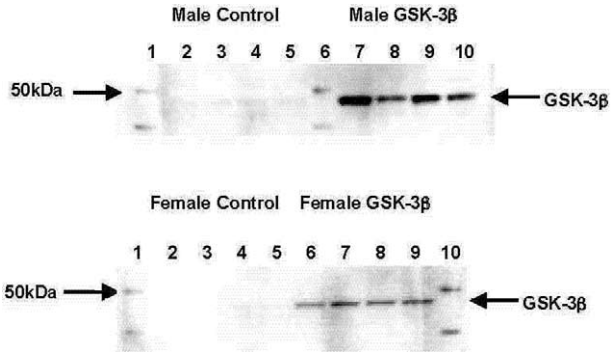

Fig 1. Western blot analysis of GSK-3β protein in gastrocnemius/plantaris muscle of (A) male and (B) female control and transgenic mice. Each lane is from 1 animal and contains 30 μg protein per lane, except for lanes 1 and 6 (A) and lanes 1 and 10 (B), which contain molecular weight markers. Each blot is from 2 to 3 representative blots each including 4 animals per group.

samples was measured on a Cobas Mira Plus clinical chemistry analyser (Roche Diagnostics, East Sussex, UK) using a glucose hexokinase assay kit (ABX Diagnostics, Bedfordshire, UK, HK125-A11A00116). Any glucose determined in the absence of α-amyloglucosidase was subtracted from that determined in the presence of α-amyloglucosidase and the amount of glycogen in each tissue expressed as milligrams glucose per gram tissue wet weight.

Oral Glucose Tolerance Test and Metabolite Measurements

Oral glucose tolerance tests were performed in mice fasted for 6 hours (10-week-old mice) or overnight (24-week-old mice).[25] Tail-tip blood (10 μL) was sampled immediately before and at 45, 90, and 135 minutes following an oral glucose (3 g/kg) load. The blood samples were hemolyzed in 1 mL of digitonin-maleimide (1 mmol/L) and glucose determined by the hexokinase assay as described above. Glucose disposal was determined as the area under the glucose tolerance curve with time, calculated using the trapezoidal rule. The concentration of insulin in plasma samples, isolated from 50-μL blood samples taken immediately before and 45 minutes after the oral glucose load, was measured using a rat insulin enzyme-linked immunosorbent assay (ELISA) kit from Crystal Chem Inc (Chicago, IL, INSKR020). ABX Diagnostics kits were used for plasma cholesterol (A11A00051/53) and triglyceride (A11A00069/71) measurement and a WAKO Chemicals USA (Neuss, Germany) kit (994-75409 E) was used for nonesterified fatty acids measurement on a Cobas Mira Plus clinical chemistry analyser (Roche Diagnostics).

Statistical Analysis

Data were analyzed by 2-way analysis of variance (ANOVA) using Statistica version 5.1 (Statsoft Inc, Tulsa, OK) and are expressed as means ± SE.

RESULTS

Overexpression of Human GSK-3β in Skeletal Muscle of Transgenic Mice

Abundant expression of human GSK-3β mRNA was detected in both the gastrocnemius/plantaris and soleus skeletal muscles of the GSK-3β transgenic mice (n = 4 for each sex). A lower level of expression of human GSK-3β mRNA was also detected in the heart (4% of that in gastrocnemius/plantaris muscle; n = 4), whereas little or no human GSK-3β mRNA was detected in liver, white or brown adipose tissue, brain, or colon (n = 2). No human GSK-3β mRNA was detected in tissues from nontransgenic animals. Levels of total GSK-3β protein were significantly higher, by 5-fold and 7-fold (both $P < .001$), in the skeletal muscles (gastrocnemius plus plantaris) of the male and female transgenic mice, respectively, compared to the control mice (Fig 1). Consistent with human GSK-3β mRNA expression in the transgenic mice, a small level of overexpression of GSK-3 protein was also detected in heart muscle (2.5-fold elevation), but not in liver, white adipose tissue (subcutaneous and perigenital), or brown adipose tissue of the transgenic mice (n = 3).

Body Weight and Composition

The GSK-3β transgenic mice gained more weight than their respective controls during the study period. At 6 weeks of age, both male and female GSK-3β transgenic mice were heavier (8% and 10%, respectively) than their respective age-matched controls (Fig 2). By 28 weeks of age, the male and female

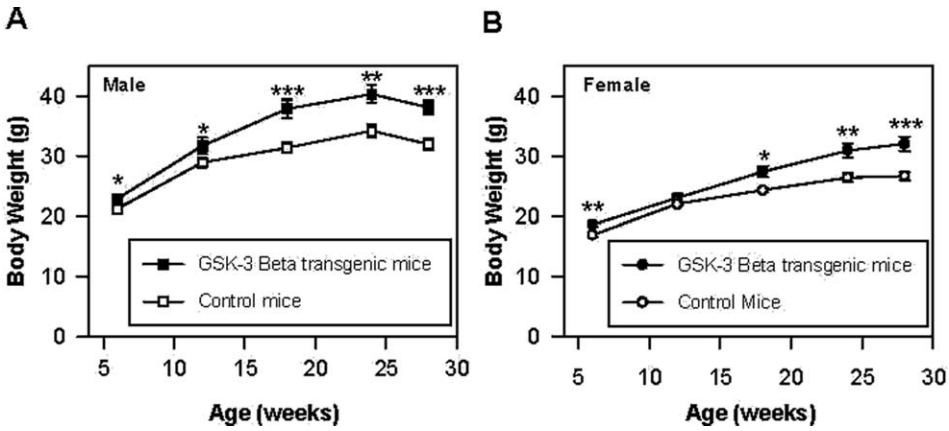

Fig 2. Body weight of transgenic and control mice between 6 and 28 weeks of age. (A) Male transgenic and control mice (n = 8 per group). (B) Female transgenic and control mice (n = 12 to 13 per group). Data are means ± SE. *$P < .05$, **$P < .01$, ***$P < .001$ for control *v* transgenic mice of same sex.

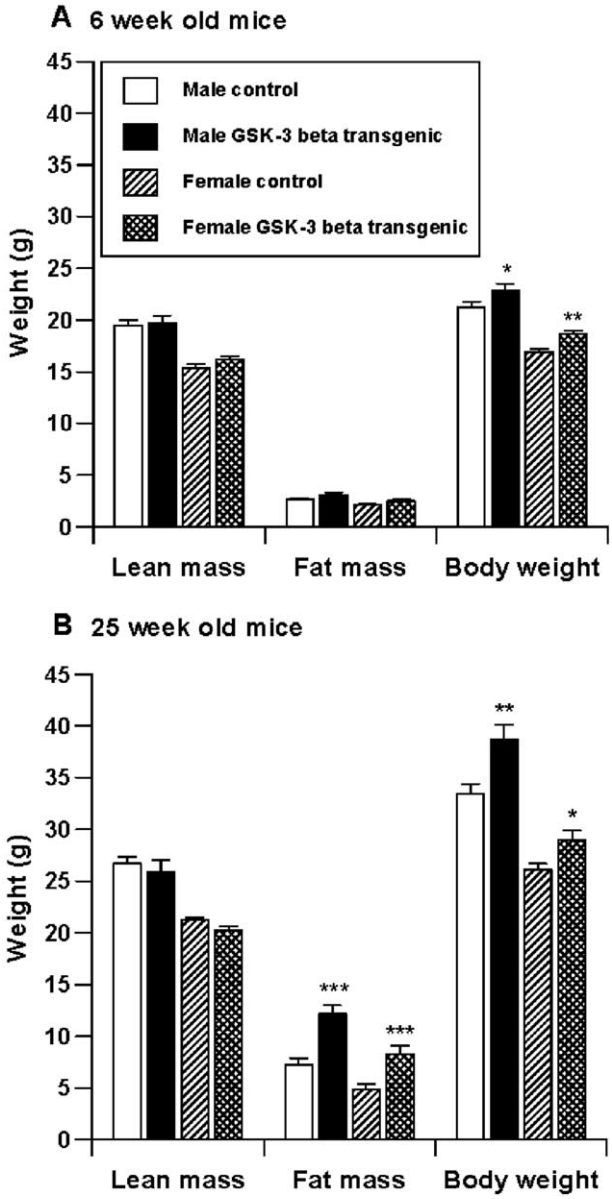

Fig 3. Body composition of transgenic and control mice at (A) 6 weeks of age and (B) 25 weeks of age. Data are means ± SE of 6 to 13 mice per group (n = 6 for lean mass and fat mass for 6-week-old mice). *$P < .05$, **$P < .01$, ***$P < .001$ for control *v* transgenic mice of same sex.

GSK-3β transgenic mice were 19% and 20% heavier than their respective controls.

The DEXA technique has been used successfully to analyze changes in body fat content (adiposity) in mice.[26] At 6 weeks of age, neither the lean mass nor the fat mass of either the male or female transgenic mice was significantly different from controls, even though their total body weights were slightly higher (Fig 3). By 25 weeks of age the fat mass, but not the lean mass, was significantly higher in both male (by 68%) and female (by 70%) transgenic mice compared to their respective controls. At termination (29 weeks of age), there was no significant difference in heart weight, but the weight of the liver, and the subcutaneous white and interscapular brown adipose depots were increased in both male and female GSK-3β transgenic animals (results not shown).

IRS-1 Protein Levels

Skeletal muscle IRS-1 protein levels were significantly lower (−67%; $P < .001$) in 29-week-old male GSK-3β transgenic mice compared to controls (Fig 4). No reduction in muscle IRS-1 levels was detected in female GSK-3β transgenic mice (98% of control value).

Glycogen Synthase Activation and Glycogen Levels in Muscle and Liver

Glycogen synthase enzyme activation, determined as the ratio of activity in the presence and absence of glucose 6-phosphate (20 mmol/L), was significantly reduced in the skeletal muscle of both male (−27%) and female (−20%) GSK-3β transgenic mice (Fig 5). There was no significant reduction in hepatic glycogen synthase activation. Skeletal muscle glycogen levels were also significantly reduced in both the male and female transgenic mice, by 58% and 55%, respectively (Fig 6). In contrast, hepatic glycogen levels were significantly increased in both the male and female transgenic mice, by 3.9-fold and 5.6-fold, respectively. By comparison with these marked differences between transgenic and control animals, there were no differences in skeletal muscle or hepatic glycogen levels between the background strains C57 Bl/6 and CBA and the hybrid B6CBAF1 strain.

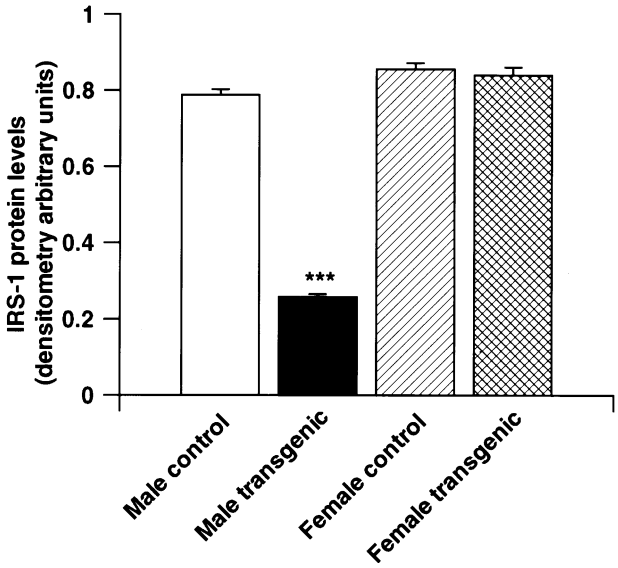

Fig 4. Western blot analysis of IRS-1 protein in skeletal muscle (gastrocnemius/plantaris), from 29-week-old male and female control and GSK-3β transgenic mice. Data are means ± SE for 7 mice per group. Blots were processed as described in the Methods and analyzed by densitometry. ***$P < .001$ for control *v* transgenic mice of same sex.

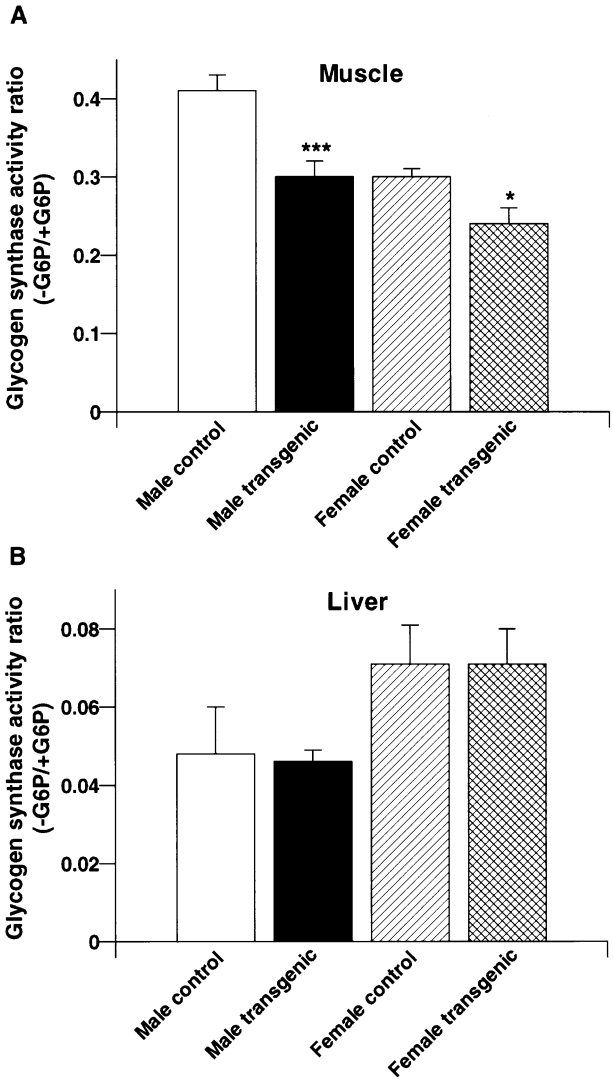

Fig 5. Glycogen synthase activity ratio (±glucose 6-phosphate 20 mmol/L) in (A) skeletal muscle (gastrocnemius/plantaris) and (B) liver of male and female control and transgenic mice aged 29 weeks. Data are means ± SE for 8 to 13 mice per group. *$P < .05$, ***$P < .001$ for control *v* transgenic mice of same age and sex.

Glucose Tolerance and Blood Lipids

The area under the oral glucose tolerance curve was not significantly increased in either male or female GSK-3β transgenic mice at 10 weeks of age (Fig 7), but the plasma insulin concentration was raised in male mice both before and 45 minutes after the glucose load (Table 1). At 24 weeks of age, fasting plasma insulin was again increased (Table 2) and the area under the glucose tolerance curve was 50% higher in the male transgenic mice than in male control mice (Fig 7). In contrast, in females, fasting plasma insulin and the area under the glucose tolerance curve were unchanged.

By contrast with these marked increases in the area under the oral glucose tolerance curve and in insulin levels in the male GSK-3β transgenic mice, the background strain and their hybrid showed no differences. The trend was for the insulin levels in the hybrid strain to actually be lower than that of the C57BL strain, while the area under the glucose tolerance curve at 24 weeks of age was only 7% higher in the hybrid strain than in the C57BL/6 strain.

Plasma cholesterol levels were significantly increased in both male and female GSK-3β transgenic mice (by 90% and 133%, respectively) at 24 weeks of age (Table 2). Plasma nonesterified fatty acids (NEFA) and triglyceride levels were also significantly increased in the male transgenic mice (by 55% and 170%, respectively).

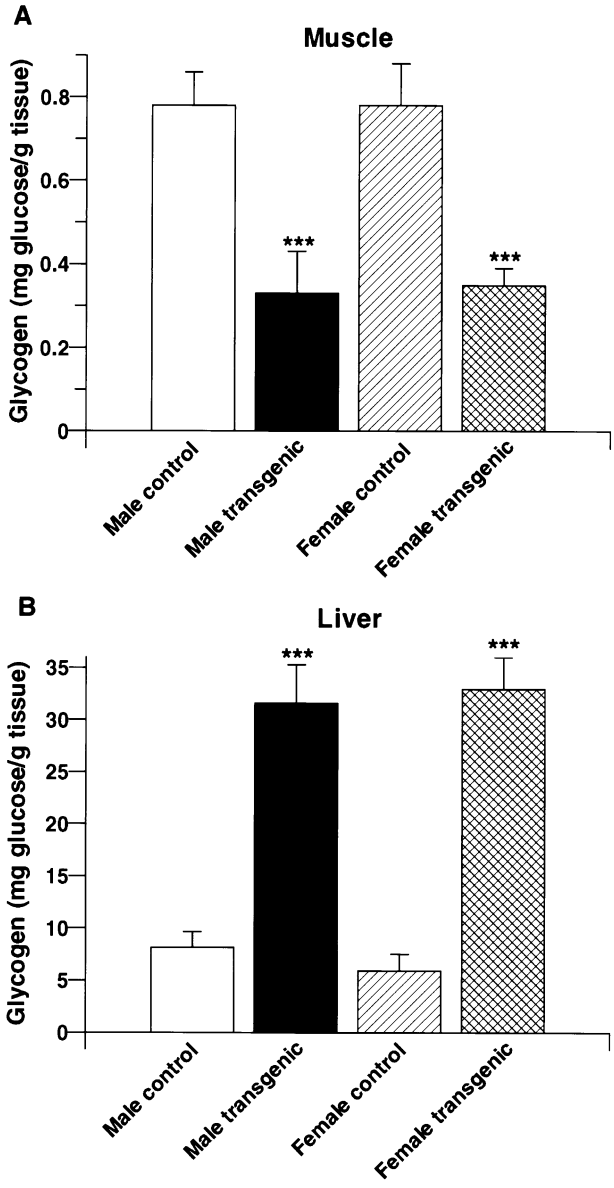

Fig 6. Glycogen levels in (A) skeletal muscle (gastrocnemius/plantaris) and (B) liver of male and female control and transgenic mice aged 29 weeks. Data are means ± SE for 8 to 13 mice per group. ***$P < .001$ for control *v* transgenic mice of same age and sex.

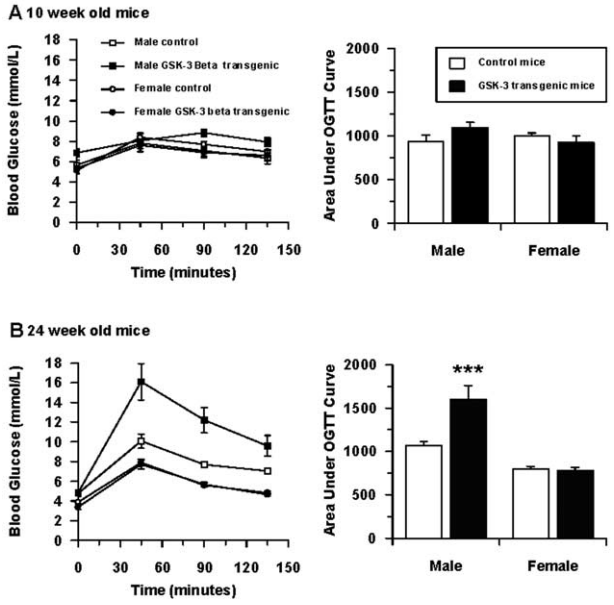

Fig 7. Effect of an oral glucose challenge on blood glucose levels in (A) 10-week-old and (B) 24-week-old transgenic and control mice. Data are means ± SE for 8 to 13 mice per group. ***$P < .001$ for control *v* transgenic mice of same sex.

DISCUSSION

Protein levels and activity of both GSK-3α and GSK-3β are elevated in the skeletal muscle of type 2 diabetic humans.[4] To investigate whether these differences might contribute to insulin resistance and glucose intolerance in type 2 diabetes, transgenic mice have been generated that overexpress human GSK-3β in their skeletal muscle. There was a 5- to 7-fold increase in skeletal muscle GSK-3β protein levels and glycogen synthase activation was significantly reduced in both the male and female mice. No overexpression of GSK-3 was observed in other organs of the GSK-3β transgenic mice (liver, white and brown adipose tissue), except for the heart, where there was a 2.5-fold increase in GSK-3 protein level.

The key feature of the GSK-3β transgenic mice was that the males became glucose-intolerant by 24 weeks of age, despite increased plasma insulin levels. At the earlier age of 10 weeks, the male transgenic mice were not glucose-intolerant, but their insulin level was raised both before and during the glucose tolerance test, suggesting that they were insulin-resistant. Additional features were reduced muscle and increased liver glycogen, increased body weight due to increased fat, increased liver and adipose tissue depot weights, and increased plasma cholesterol, NEFA, and triglyceride levels. This phenotype is similar to that described in a patent application[27] for another line of mice that overexpresses GSK-3β in skeletal muscle. The phenotype suggests that increased GSK-3 activity in the skeletal muscle of type 2 diabetic humans[4] might contribute to their insulin resistance and glucose intolerance.

Although glycogen synthase activation was equally reduced in muscle of both male and female mice, physiologically, the phenotype of the females was not as marked as that of the males. The females were obese and had reduced muscle and increased liver glycogen, but they were not glucose-intolerant; nor were their plasma NEFA levels significantly elevated. Such differences between male and female rodents are common findings. For example, several other types of male transgenic mice have also shown a greater tendency to develop glucose intolerance, insulin resistance, and diabetes than their female counterparts,[28-30] suggesting that male mice are generally more susceptible to insulin resistance. The molecular mechanisms underlying this difference are not understood.[31] Not only did the female transgenic mice not have elevated plasma NEFA levels, but also IRS-1 protein levels were not reduced in skeletal muscle. These could be key links between GSK-3 and impaired glucose tolerance that were missing in the females. On the other hand, both elevated NEFA and lowered IRS-1 levels could be consequences of insulin resistance produced by other mechanisms in the males but not in the females. For the remainder of this discussion, in common with most other workers, we will focus on our findings in male mice. We nevertheless acknowledge that further studies comparing females with males are needed, not just in GSK-3 transgenic mice, but also in other genetically modified mice, since these may provide the key to a full understanding of insulin resistance.

The mechanism by which increased GSK-3 activity produces glucose intolerance is probably multifactorial. In addition to directly influencing insulin signaling, the obesity of the GSK-3β transgenic mice coupled with high NEFA levels in males (possibly indicating high intracellular fatty acid metabolite levels) may have contributed to their glucose intolerance. However, the molecular mechanisms responsible for these changes are obscure, whereas mechanisms that might link GSK-3 more directly with insulin action are well known.

Increased GSK-3 activity may cause glucose intolerance independent of obesity by serine phosphorylation and inhibition of the activity of glycogen synthase.[3,32] Consistent with this explanation, glycogen synthase activation and glycogen levels were lower in skeletal muscle of male GSK-3β transgenic mice than in wild-type mice. Similarly in humans, glycogen synthase activity was low in the skeletal muscle of insulin resistant and diabetic subjects[4,33,34] and skeletal muscle glycogen synthase activity was negatively correlated with both GSK-3α and GSK-3β protein expression.[4] Furthermore, the time course of GSK-3 deactivation and glycogen synthase activation were almost identical during physiological insulin

Table 1. Plasma Insulin During the Glucose Tolerance Test at 10 Weeks of Age

	0 Minutes (ng/mL)	45 Minutes (ng/mL)
Male control	0.43 ± 0.07	1.04 ± 0.17
Male GSK-3β transgenic	1.45 ± 0.27†	1.83 ± 0.54*
Female Control	0.24 ± 0.04	0.66 ± 0.07
Female GSK-3β transgenic	0.50 ± 0.09	0.85 ± 0.14

NOTE. The mice had been fasted for 6 hours. Blood was taken before and during the glucose tolerance test shown in Fig 7A. Data are means ± SEM for 8 to 12 mice per group.

*$P < .05$, †$P < .001$ for control *v* transgenic mice of same sex.

Table 2. Plasma Insulin, NEFA, Triglyceride, and Cholesterol Levels at 24 Weeks of Age

	Insulin (ng/mL)	NEFA (mmol/L)	Triglyceride (mmol/L)	Cholesterol (mmol/L)
Male control	0.45 ± 0.04	2.06 ± 0.27	0.63 ± 0.15	3.37 ± 0.59
Male GSK-3β transgenic	0.75 ± 0.14‡	3.20 ± 0.28*	1.70 ± 0.30‡	6.40 ± 1.14†
Female control	0.37 ± 0.04	2.04 ± 0.32	0.71 ± 0.13	1.73 ± 0.19
Female GSK-3β transgenic	0.41 ± 0.04	2.40 ± 0.22	0.89 ± 0.13	4.04 ± 0.65†

NOTE. The mice had been fasted overnight. Data are means ± SEM for 4 to 13 mice per group.
*$P < .05$, †$P < .01$, ‡$P < .001$ for control *v* transgenic mice of same age and sex.

clamp conditions in humans.[35] It is possible that in humans reduced glycogen synthase activity is also due in part to its reduced dephosphorylation and activation by the phosphatase PP1G.[36]

Consistent with the findings in other animal models of insulin resistance,[37,38] skeletal muscle IRS-1 protein levels were reduced in male GSK-3β transgenic mice. Since IRS-1 plays a pivotal role in mediating insulin signaling, the reduced smooth muscle IRS-1 levels in male transgenic mice could, at least in part, explain why they became glucose-intolerant. The link between raised skeletal muscle GSK-3β protein levels and reduced IRS-1 levels in these mice is clearly of interest. Reduced IRS-1 levels in male transgenic mice could have been due to raised insulin levels, since insulin is believed to induce IRS-1 degradation through stimulation of IRS-1 serine/threonine phosphorylation, possibly via the phosphatidylinositol 3 kinase and mTOR pathways.[39] Other diabetogenic factors, such as the raised NEFA levels seen only in male GSK-3β transgenic mice, may also inhibit IRS-1 activity.[18] Alternatively, GSK-3 could directly stimulate IRS-1 phosphorylation and degradation.[16] Consistent with this latter hypothesis, chronic treatment of human skeletal muscle cells with GSK-3 inhibitors led to a decrease in GSK-3 protein and activity, but an increase in IRS-1 protein and insulin-stimulated glucose uptake.[40]

Skeletal muscle glycogen levels were reduced by half in transgenic mice. This was presumably a consequence of reduced glycogen synthase activity; overexpression of muscle glycogen synthase in mice has conversely been shown to be associated with increased glycogen.[10] By contrast, in type 2 diabetic humans, muscle glycogen levels are maintained, presumably by hyperglycemia and hyperinsulinemia.[41] There is therefore not a consistent relationship between muscle glycogen levels and the sensitivity of muscle glucose uptake to insulin.

Whereas muscle glycogen levels were reduced, hepatic glycogen levels were increased by 4- to 6-fold. The liver thus appears to compensate, at least in part, for the reduced muscle glycogen storage. Although high hepatic glycogen concentrations can reduce glycogen synthase activity,[42] there was no difference in hepatic glycogen synthase activation between the transgenic and control mice. Increased liver glycogen storage does not appear to be due to increased exposure of the liver to blood glucose and insulin because these were not raised in female transgenic mice. A possible alternative explanation is that glucose that cannot be stored as muscle glycogen is converted to gluconeogenic substrates.

Interestingly, male mice that lack insulin receptors in muscle also have compensatory mechanisms that ameliorate the impact of muscle insulin resistance on glucose homeostasis.[43] One mechanism in these mice is enhanced insulin-stimulated glucose transport into white adipose tissue in vivo, despite glucose transport into isolated white adipocytes being normal. These mice are also obese.

In addition to demonstrating that raised GSK-3 activity in skeletal muscle may contribute to insulin resistance in type 2 diabetes, the present study suggests that GSK-3 inhibitors may be effective in the treatment of this disease. GSK-3 is an attractive target for drugs. It has the advantage that inhibitors rather than activators are required; indeed, selective inhibitors have already been described that stimulate glycogen synthesis in human liver and skeletal muscle cells.[22,40,44] We have focused on the role of GSK-3 in muscle, but inhibition of GSK-3 in liver may also contribute to blood glucose control, either by promoting glycogen synthesis, which may also reduce gluconeogenesis,[45] or because GSK-3 inhibition in liver reduces the expression of key gluconeogenic enzymes.[46] Indeed Cline et al[47] have reported that 2 small-molecule GSK-3 inhibitors improved glucose tolerance in male Zucker diabetic fatty rats primarily by promoting liver glycogen synthesis. Muscle glucose uptake and glycogen synthesis were not significantly increased by GSK-3 inhibition despite activation of glycogen synthase. Other studies, however, have found that GSK-3 inhibitors can enhance glucose uptake in skeletal muscle.[40,48,49] In one study, GSK-3 inhibition was effective in Zucker diabetic fatty rats, in which GSK-3 activity is elevated, but not in normal rats.[49] Thus, there may be situations in which inhibiting GSK-3 cannot promote muscle glucose utilization above normal levels, whereas increasing the activity of GSK-3, as in our study, can inhibit muscle glucose utilization. Although our results suggest that GSK-3 inhibitors may only be effective in males, others have reported that they are effective in both male and female rodent models of diabetes.[50]

In summary, the results presented here demonstrate that male transgenic mice overexpressing GSK-3β in their skeletal muscle become glucose-intolerant, despite raised plasma insulin levels. In addition these mice exhibit other characteristics of impaired peripheral glucose metabolism in humans, such as reduced muscle glycogen synthase activity, reduced muscle IRS-1 protein levels, and increased plasma cholesterol, NEFA, and triglyceride levels. The phenotype of these mice, along with the increased expression of GSK-3 and lower glycogen

synthase activity observed in the muscle of diabetic patients,[4] suggests a role for skeletal muscle GSK-3 in the pathogenesis of insulin resistance and glucose intolerance. These results also suggest that selective inhibitors of this enzyme, similar to those described previously,[22,44,47,49-52] may have therapeutic potential in the treatment of insulin resistance and diabetes. Further studies are needed to investigate why female transgenic mice were not glucose-intolerant.

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