Moderate GLUT4 overexpression improves insulin sensitivity and fasting triglyceridemia in high fat fed transgenic mice

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ABSTRACT

The GLUT4 facilitative glucose transporter mediates insulin-dependent glucose uptake. Herein, we test the hypothesis that moderate overexpression of human GLUT4 in mice, under the regulation of the human GLUT4 promoter, can prevent hyperinsulinemia resulting from obesity. Transgenic mice engineered to express the human GLUT4 gene and promoter (hGLUT4 TG) and their non-transgenic counterparts (NT) were fed either control diet (CD) or high fat diet (HFD) for up to ten weeks. HOMA-IR scores revealed that hGLUT4 TG mice remained highly insulin sensitive on HFD. Presence of the GLUT4 transgene did not completely prevent the metabolic adaptations to HFD. For example, HFD resulted in loss of dynamic regulation of several metabolic genes expression in the livers of fasted and refed NT and hGLUT4 TG. The hGLUT4 TG mice on CD showed no feeding-dependent regulation of SREBp-1c and fatty acid synthase (FAS) mRNA expression in the transition from the fasted to the fed state. Similarly, HFD altered the response of SREBP-1C and FAS mRNA expression to feeding in both strains. These changes in hepatic gene expression were accompanied by increased nuclear phosphoCREB in refed mice. Taken together, a moderate increase in expression of GLUT4 is a good target for treatment of insulin resistance.
INTRODUCTION

GLUT4, the insulin-responsive facilitative glucose transporter, is expressed in adipose, skeletal muscle and cardiac muscle cells. GLUT4 expression levels are correlated with whole body insulin-mediated glucose homeostasis. Two lines of evidence supported this notion. First, enhanced insulin-sensitivity after exercise is associated with increased GLUT4 expression in skeletal muscle (1; 2). Second, transgenic manipulation of GLUT4 in mice revealed a profound effect on both glucose and lipid homeostasis (3-6). In addition, insulin-resistant glucose transport in adipocytes from obese and diabetic subject correlated with reduced GLUT4 mRNA and protein (7-9), confirming a role of GLUT4 for insulin-dependent glucose homeostasis.

Adipose tissue and skeletal muscle play unique roles in the regulation of insulin-dependent glucose homeostasis. Proliferation of adipose mass is tightly linked to obesity and development of insulin resistance. Expansion of adipose mass in obesity is associated with decreased GLUT4 mRNA and protein expression leading to insulin-resistant glucose transport (10; 11). The fat-specific knockout of GLUT4 impacts whole body glucose homeostasis and leads to insulin resistance in muscle and liver (12). This clearly demonstrated a central role for adipose tissue, and GLUT4 levels in adipose tissue to impact metabolic control. It is not clear why nutrient excess leads to a loss of GLUT4 in adipose tissue, and it is also clear that overexpression of GLUT4 only in adipose tissue is not sufficient to protect against diet-induced glucose intolerance (13).
Skeletal muscle is the major site of dietary glucose disposal in the body (14). In insulin resistant states, glucose transport into skeletal muscle was impaired (15; 16). At the outset, skeletal muscle insulin resistance occurs through inhibition of GLUT4 redistribution to the cell surface (17; 18), indicating a distinct mechanism for regulation of GLUT4 in muscle tissue compared to adipose tissue. In severe insulin resistance, muscle GLUT4 protein and mRNA can be reduced similar to adipose tissue (19). Muscle-specific transgenic expression of GLUT4 improved insulin action in diabetic mice (20; 21), which may be attributable to enhanced basal accumulation GLUT4 at the cell surface, as well as a partial correction of the defect in insulin-mediated GLUT4 translocation (21). Conversely, transgenic muscle-specific ablation of GLUT4 results in insulin resistance and inadequate glucose tolerance (22). Taken together, GLUT4-dependent glucose transport in skeletal muscle is likely the major mechanism for dietary glucose disposal by skeletal muscle.

Whole body insulin-dependent glucose homeostasis results from the interactions of several tissues including the pancreatic beta cells, liver, adipose tissue and skeletal muscle. Transgenic mouse models have demonstrated clearly that manipulation of insulin action in one tissue can impact function of other tissues, making it difficult to identify any one target for prevention and treatment of type 2 diabetes (23). Herein, we used transgenic mice that moderately over-express the human GLUT4 gene under the control of its own promoter, as previously described (4; 24), to better understand the physiologic role that GLUT4 can play in protection against insulin-resistant glucose homeostasis. This line of
transgenic mice, referred to as hGLUT4 TG mice, has two unique features that set them apart from other models. First, the level of over-expression is two to three-fold over the endogenous GLUT4, which is similar to increased expression that might be attained from a long-term exercise program (25; 26). Second, the transgene is driven by a dynamic human GLUT4 promoter, allowing the transgene to undergo physiologic regulation (4; 27). Using this model, we demonstrated that this level of moderate GLUT4 over-expression is highly protective against the development of peripheral insulin resistance in response to diet-induced obesity.

**MATERIALS AND METHODS**

*Animals and Diets*

Animals used for these experiments were male C57Bl/6 mice carrying a random insertion of the human GLUT4 gene (hGLUT4 TG) have been described (4; 27). The non-transgenic control mice (NT) are the non-transgenic littermates. All procedures using animals were approved by the Institutional Animal Care and Use Committee at the University Of Oklahoma Health Sciences Center.

All mice were kept in a temperature-controlled room with a 12-hour light/dark cycle. Eight to ten weeks after birth the mice were housed in individual cages, fasted 17 hr for measurement of initial fasting parameters, and ad libitum fed either control diet (CD) (10% kcal from fat, D12450B) or high fat diet (HFD) (60% kcal from fat, D12492) from Research Diets Inc. (New Brunswick, NJ) for indicated times.

*Food Consumption Measurement*
Food consumption was estimated weekly by subtracting the mass of food left from the initial mass of food supplied. Energy intakes were calculated on the basis of 3.8 kcal/g for CD and 5.2 kcal/g for HFD.

**Blood and Plasma Assays**

Blood was collected after 0, 4, and 8 weeks of feeding. The mice were fasted for 17 hours, and blood samples from tail-veins of conscious mice were collected in tubes containing EDTA. Plasma insulin concentrations were determined using the Ultra-Sensitive Mouse Insulin ELISA Kit (Crystal Chem, Downers Grove, IL). Fasting plasma leptin concentrations were determined using the Mouse Leptin ELISA Kit (Crystal Chem). Blood glucose was measured using the TrueTrack glucometer.

**HOMA-IR scores**

The homeostasis model assessment of insulin resistance (HOMA-IR) scores were calculated using fasting glucose and fasting insulin concentrations obtained from mice after 17 hours of fasting, and using the following formula: fasting blood glucose (mg/dl) × fasting insulin (µU/ml)/405.

**Body mass and body composition measurements**

Body mass and body composition was measured using a Minispec Body Composition analyzer (Bruker, LF-90). Measurements were made weekly between 10 am and noon on non-fasted mice.

**Pyruvate tolerance test**
Following an overnight fast, mice were given an intraperitoneal dose of 2 g/kg sodium pyruvate. Blood glucose levels were measured 0, 20, 40, 60, 90 and 120 minutes post injection.

Protein Analysis

Western blot analysis to estimate GLUT4 protein concentration was completed for epididymal white adipose tissue (WAT), subscapular brown adipose (BAT) and quadriceps femoris skeletal muscles (SKM). Total detergent extracts were prepared in lysis buffer containing 20 mM HEPES, 2% NP40, 2 mM EDTA, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1mM molybdate, protease inhibitor cocktail (complete Mini EDTA-free Protease Inhibitor Cocktail, Roche), and 1 mM PMSF. Liver nuclear extracts were obtained using a kit (Pierce). Protein concentrations were determined by using a Coomassie Plus (Bradford) Assay Kit (Pierce). Lysates were fractionated using 10% SDS-PAGE, and proteins were transferred to an Immobilon-FL polyvinylidene fluoride membrane (EMD Millipore Corporation, Billerica, MA, US.) Membranes were stained with anti-GLUT4 antibody (C-20 goat polyclonal antibody, Santa Cruz Biotech), total Akt antibody (Cell Signaling), phosphoCREB (Cell Signaling) or total CREB (Cell Signaling) and visualized with appropriate secondary antibodies conjugated with AlexaFluor 680. Fluorescence was quantified Li-Cor Odyssey imager (Li-Cor Biosciences, USA).

RNA extraction, quantitative real-time PCR

Mice were fasted for 17 hours or were fasted for 17 hours then refed for 4 hours. Following the fasting or fasting/refeeding, animals were killed, and tissues were
harvested and snap frozen in liquid nitrogen. Tissues were stored at -80°C until used later. Messenger RNA (mRNA) was extracted as previously described (28). Samples were stored as an ethanol precipitate at -20°C until further analysis. mRNA levels of mouse GLUT4, human GLUT4, PEPCK, G6Pase, SREBP-1c, FAS, HMG CoA Reductase were quantified by quantitative real-time (qrt) PCR. Primer sequences were as follows: mouse GLUT4, 5' -

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AAAAGTGCCTGAACACCAGAG-3' (forward), 5' -TCACCTCCTGCTCTAAAGG-3' (reverse); human GLUT4 5' -GCGGCAGATGAAAGAAG-3' (forward), 5' -CTCCAGGCGGAGTCAGA-3' (reverse); UCP-1, 5' -GTGAGGTCAGAATGCAAGC-3' (forward), 5' -aGGGCCCTCTTATGAGGTG-3' (reverse); UCP-2, 5' -CAGCCA GCC CCA AGT ACC-3' (forward), 5' -CAA TGC GGA CGG AGG CAA AGC-3' (reverse); PEPCK, 5' -ACACACACACATGCTCACAC- 3' (forward), 5' -ATCACCGCATAGTCTCTGAA- 3' (reverse); G6Pase, 5' -TGGTAGCCCTGTCTTTCTTT-3' (forward), 5' -TTCCAGCATTACACTTTTCTCT-3' (reverse); SREBP-1c, 5' -GTGAGCCTGACAAGCAATCA- 3' (forward), 5' -GGTGCCTACAGAGCAAGAG-3' (reverse); FAS, 5' -GCTGCGGAACTTCACTGAAATG-3' (forward); TBP, 5' -GAAGCTGCGGTACAATTCCAGT-3' (forward), 5' -CCCCTGTACCCCTCACCAAT-3' (reverse). The relative mRNA levels were calculated using a standard curve developed from a normal mouse tissues. All qrt-PCR were completed using a modular thermal cycler platform composed of a C1000 Touch™ thermal cycler chassis (Bio-rad Laboratory, Inc.) and a CFX96™ optical reaction module (Bio-rad
Laboratory, Inc.). Data from qrt-PCR was read using CFX Manager™ software (Bio-rad Laboratory, Inc.).

**Triacylglycerol analysis**

Plasma triacylglycerol was measured using the triglyceride determination kit (Sigma) according to manufacturer's specifications. Liver triacylglycerol levels were measured using the same kit, after a folch extraction of lipids, and resuspension in 0.1% triton-X100.

**Statistical analysis**

Data were expressed as mean and standard error of the mean. All statistical analysis was completed using VassarStats web-based software. Comparisons of groups were carried out using 1-way or 2-way ANOVA against mice fed a CD or NT mice as the control groups. Pairwise comparisons were made using confidence interval set at 95%. Unless otherwise noted significance was p < 0.05.

**RESULTS**

*Moderate GLUT4 expression protects against diet-induced insulin resistance.*

To evaluate the effects of moderate hGLUT4 overexpression under conditions of HFD feeding we measured blood glucose and plasma insulin levels in mice carrying the human GLUT4 gene under the control of the human GLUT4 promoter (hGLUT4 TG) and their non-transgenic littermate controls (NT). Blood glucose levels of hGLUT4 TG mice were significantly lower than NT mice fed both CD and HFD (Table 1). In NT mice, the blood glucose levels were significantly increased after 4 and 8 weeks on HFD compared to 0 weeks. Blood glucose levels
in hGLUT4 TG mice on HFD were increased at 8 weeks, but still significantly lower than NT counterparts on HFD.

Similar to blood glucose levels, fasting insulin levels were lower in hGLUT4 TG compared to NT under both dietary regimens at each time point (Table 1). The HFD regimen increased fasting plasma insulin levels in NT animals at both 4 and 8 weeks, and in hGLUT4 TG at 8 weeks only.

The fasting blood glucose level and plasma insulin level data are consistent with the notion that hGLUT4 TG mice are less predisposed to HFD-induced insulin resistance. To quantify insulin resistance, we used the homeostasis model assessment of insulin resistance (HOMA-IR), hGLUT4 TG mice fed CD had a significantly lower average HOMA-IR score than NT mice fed the CD at all time points (Figure 1). Throughout HFD feeding, the HOMA-IR score for hGLUT4 TG mice was significantly lower than the score for NT mice. These data indicate that moderate hGLUT4 overexpression in mice significantly reduced development of HFD-induced insulin resistance.

*Moderate GLUT4 overexpression increases food intake, but not adiposity*

To determine if moderate hGLUT4 overexpression affects HFD-induced body composition, body mass, adipose mass and lean mass were measured in both strains of mice fed either CD or HFD for 8 weeks. HFD conditions significantly increased both body mass and adipose mass in both strains of mice (Figure 2A). The change in adipose mass was reflective of increases in white adipose pads but not brown adipose (Figure 2B). Although total adiposity is not different between hGLUT4 TG and NT mice fed HFD, the mass of the epididymal
fat pad of TG mice was significantly greater when expressed as a percentage of body mass (Figure 2B).

The increase in adipose mass was accompanied by increased food intake over the 8-week feeding period. HFD increased energy intake in both NT and hGLUT4 TG mice compared to their CD-fed counterparts (Figure 3A). When fed HFD, the hGLUT4 TG mice consumed on average 10% more kcal/week than NT mice (Figure 3A). While the 10% increase in food intake did increased epididymal fat pad size (Figure 2B), this increase in food intake was not sufficient to result in a measurable increase in total adiposity in TG compared to NT mice fed HFD (Figure 2A). The increased food intake may be partially offset by changes in UCP-1 expression in the visceral fat pad. This is supported by the observation that HFD feeding specifically reduced UCP-1m RNA, but not UCP-2 mRNA in epididymal fat of NT mice fed HFD (Figure 3B and 3C). In both strains, the increase in adipose mass correlated with increased plasma leptin levels (Figure 3D). Plasma leptin levels rose as a function of time on HFD for both strains; however, leptin levels were significantly lower in hGLUT4 TG at 4 and 8 weeks on HFD compared to NT on HFD (Figure 3D).

*Moderate GLUT4 overexpression does not prevent loss of GLUT4 expression in adipose tissue*

The blood glucose, plasma insulin, and HOMA-IR data suggest that hGLUT4 TG mice may be protected from insulin resistance presumably through enhanced glucose uptake as a result of mild overexpression of human GLUT4 in peripheral tissues (4; 6). However, it is not clear which tissues are responsible for
this effect. To determine if HFD induced reductions in GLUT4 expression is modified in white adipose tissue (WAT), brown adipose tissue (BAT) and skeletal muscle (SKM) in hGLUT4 TG, immunoblot analysis of GLUT4 levels in these tissues was carried out after 10 weeks on CD or HFD. As expected, immunoblot analysis indicated hGLUT4 TG mice fed the CD had 2 to 3-fold higher GLUT4 expression in WAT, BAT and SKM compared to NT mice fed the CD (Figure 4) (4; 24). When fed the HFD, GLUT4 expression in WAT decreased to similar levels in both strains (Figure 4A). The changes in GLUT4 protein were correlated with changes in GLUT4 mRNA (Figure 4B). Both endogenous mouse GLUT4 mRNA and transgenic mRNA were similarly down-regulated in response the HFD. HFD feeding reduced GLUT4 levels in BAT, however, the GLUT4 levels HFD-fed hGLUT4 TG mice remained significantly higher than in HFD-fed NT mice (Figure 4C). The HFD feeding significantly reduced GLUT4 protein levels in skeletal muscle NT mouse, but had no effect on skeletal muscle GLUT4 in hGLUT4 TG mice (Figure 4D). Taken together, it appears that the main effects of GLUT4 overexpression may be due to enhanced glucose uptake in SKM and BAT. This is consistent with the observation that skeletal muscle was a significant reservoir for hGLUT4 TG mice fed control diet (6).

**Moderate GLUT4 overexpression does not prevent diet-dependent changes in hepatic gene expression due to high fat feeding**

While moderate overexpression of hGLUT4 appears to maintain GLUT4 in SKM and protect against peripheral insulin resistance, it is not known if it prevents all physiologic adaptations to a HFD. To test this possibility, we evaluated hepatic
expression of gluconeogenic genes in the fasted and fasted/refed states to
determine if dynamic regulation during this transition was retained. To evaluate
gene expression in the transition between fasting and refeeding, both hGLUT4 TG
and NT mice fed either CD or HFD were subjected to a 17 hr fast or a 17 hr fast
followed by 4 hr refeeding. Under refed conditions, both hGLUT4 TG and NT mice
fed CD displayed a significantly lower expression of phosphoenolpyruvate carboxy
kinase (PEPCK) and glucose-6-phosphatase (G6Pase) mRNA compared to their
fasted counterparts (Figure 5A and B). When fed HFD, both hGLUT4 TG mice and
NT mice lost dynamic regulation PEPCK or G6Pase mRNA expression when
comparing fasted and refed state (Figure 5A and B). These data demonstrate that
HFD resulted in a constitutive gluconeogenic gene expression in the liver as
previously described (29). Despite the changes in gluconeogenic gene expression
with HFD, the hGLUT4 TG mice had significantly increased tolerance to pyruvate
compared to NT mice (Figure 5C). This suggests that either hepatic
gluconeogenesis was lower or peripheral clearance of glucose was higher in the
hGLUT4 TG mice.

The loss of dynamic regulation of gluconeogenic was not correlated to diet-
induced changes in fed insulin levels, as these did not change for hGLUT4 TG
mice on CD compared to HFD (Figure 5D). Fasted and refed glucose levels were
significantly lower in hGLUT4 TG under both CD and HFD when compared NT
mice on the same diets. Fed insulin levels were indistinguishable between strains
fed CD, however, NT mice fed a high fat diet trended towards higher fed insulin
levels (Figure 5D).
To determine if HFD altered counter-regulatory hormone action in the refed state, we measured phosphoCREB and total CREB in liver nuclear extracts (Figure 5E). As expected, the ratio of phospho CREB to total CREB in liver nuclei of was higher in fasted mice compared to refed mice. This pattern was observed in both strains fed CD, and in NT met HFD. The hGLUT4 TG mice fed HFD lost the dynamic regulation of phospho CREB in the transition to from fasting to the refed state. Hepatic nuclear accumulation of phospho CREB in hGLUT4 TG mice fed was significantly lower in the fasted state and significantly higher in the refed state (Figure 5E).

The transition from fasting to refeeding is accompanied by increased SREBP-1c expression, a transcription factor required for transcription of lipogenic genes regulated by insulin (29). As expected, hepatic expression of SREBP-1c mRNA increased following refeeding of fasted NT mice fed CD (Figure 6A). SREBP-1c expression did not increase in response to refeeding in hGLUT4 TG mice fed CD (Figure 6A). When fed HFD, NT mice lost the ability to increase SREBP-1c mRNA in response to refeeding. Under both feeding regimens, SREBP-1c mRNA in refed hGLUT4 TG mice was significantly lower than in the refed NT mice, indicating that the transgene, rather than diet, was the dominant regulator of SREBP1-c mRNA expression. Changes in expression of fatty acid synthase (FAS) mRNA, a target of SREBP-1c, mirrored the diet and strain-dependent changes in observed for SREBP-1c mRNA (Figure 6B).

Fasting plasma triacylglycerol (TAG) levels were higher in hGLUT4 TG mice compared to NT mice fed control diet (Figure 6C). HFD did not alter fasting
plasma TAG levels in NT mice; however, HFD significantly reduced fasting plasma TAG levels in hGLUT4 TG (Figure 6C).

Refed plasma TAG levels were similar in all four groups of mice (Figure 6C). In the transition from fasted to fed, hGLUT4 TG mice had a significant decrease in plasma TAG, while this transition led to an increase in TAG in NT mice (Figure 6C). This strain-dependent change in plasma TAG levels from fasting to feeding may reflect the relative hypoinsulinemia present in the hGLUT4 TG mice fed the control diet. Both strains, when fed a HFD showed no significant change in plasma TAG in the transition from fasted to fed.

The liver TAG levels were subject to the expected temporal changes in the transition from fasted to refed state. In all cases, fasted liver TAG was significantly higher than refed liver TAG (Figure 6D). In all cases, refeeding lowered the liver TAG content; however, both strains had significantly higher fed liver TAG when fed HFD. Fasted liver TAG was significantly higher in hGLUT4 TG mice on control diet when compared to NT mice on the same diet. Both strains, when fed HFD, had significantly elevated fasting liver TAG in the fasted state compared to NT mice on CD.

**DISCUSSION**

HOMA-IR measurements revealed that hGLUT4 TG mice remained relatively insulin sensitive after 8 weeks on HFD, although the insulin sensitivity was slightly decreased compared to transgenic mice fed CD over the same time period. The HOMA-IR score for hGLUT4 TG mice fed the HFD stayed with in the range of scores for NT mice fed the control diet indicating that the moderate over-
expression of GLUT4 was sufficient to reduce hyperinsulinemia and hyperglycemia.

GLUT4 has been known to play a major role in regulating insulin-mediated glucose homeostasis in normal physiology. Targeted and whole-body genetic manipulation of GLUT4 levels have demonstrated that GLUT4 is limiting for glucose uptake and utilization in skeletal muscle and adipose tissue (4; 6; 12; 21; 30; 31). Changes in GLUT4 expression in adipose tissue, and insulin-dependent GLUT4 translocation in skeletal translocation in models of insulin deficiency and insulin resistance have implied a role for GLUT4 in the pathogenesis of insulin-resistance metabolic diseases (7; 9; 18; 32). Taken together, this work shows that loss of GLUT4 at the cell surface in response to insulin (either through decreased expression or decreased translocation) is correlated with insulin resistance.

Transgenic over-expression of GLUT4 either under the control of the native promoter or a tissue-specific, heterologous promoter leads to enhanced insulin sensitivity, enhanced glucose clearance, and some protection against insulin resistance. These observations have led many authors to conclude that up-regulating glucose transporters may be an effective approach for the treatment of human type 2 diabetes (11; 20; 33-36). This notion was initially challenged by the observation that transgenic expression of human GLUT4 under the control of its own promoter enhances insulin sensitivity in chow-fed mice, but at the same time significantly increased serum TAG levels, free fatty acids and ketones (6). Similarly, transgenic expression in adipose tissue, under the control of the AP2 promoter, also had increased serum triacylglycerol and free fatty acids (37). In the
current paper, we demonstrate that, when stressed with a high fat diet, mild overexpression of GLUT4 under the control of a dynamic human GLUT4 promoter protects against insulin-resistance, and results in plasma TAG in fasting and refeeding that are indistinguishable between hGLUT4 TG and NT mice. The elevated plasma TAG levels in the hGLUT4 TG fed the CD likely were a result of reduced clearance of very low-density lipoprotein (VLDL) particles as a result of the unusually low plasma insulin levels. Chronically low plasma insulin levels may reduce adipose and muscle lipoprotein lipase (LPL) activity as well as reduce VLDL receptor number (38; 39). Both strains, when fed high fat diet, developed plasma TAG levels that were undistinguishable between strains. More important, under HFD, the difference between fasting and refed plasma TAG was no longer statistically significant. The loss of regulation appeared to result from a trend toward increased fasting plasma TAG, which may reflect insulin-resistant LPL activity associated with diet-induced obesity (40).

Despite very low HOMA-IR scores, moderate overexpression of GLUT4 may not completely prevent the metabolic adaptations to a high fat diet. While hGLUT4 transgenic animals remained relatively insulin sensitive, changes in adipose GLUT4 gene expression and the changes in hepatic gluconeogenic gene expression were consistent with expected adaptations to an obesogenic diet (41; 42). Because the HFD-dependent changes in gluconeogenic gene expression were not correlated with the pyruvate tolerance test (hGLUT4 TG mice remained highly pyruvate tolerant), it is likely that enhanced insulin sensitivity was due to increased peripheral glucose uptake, presumably in skeletal muscle. This is
consistent with hyperinsulinemic clamp studies performed on these the hGLUT4 TG mice fed the obesogenic Surwit diet (34). After 8 weeks on HFD, the HOMA-IR score for hGLUT4 TG mice was increased compared to hGLUT4 TG mice on CD. This slight increase in HOMA-IR may be a reflection of decreased GLUT4 expression in adipose tissue (Figure 4A) or to potential diet-dependent changes in GLUT4 abundance at the cell surface of skeletal muscle.

Our data revealed that HFD-dependent altered gene expression in both adipose tissue and liver occurred independently of the magnitude of changes in HOMA-IR. For example, the changes in GLUT4 expression in adipose tissue decreased to a similar extent in NT and hGLUT4 TG mice even though the HOMA-IR scores were different (Figure 1 and 4B). The changes in gene expression were also not due to changes in fed insulin levels, as these values were not statistically different between groups. The changes may be due either to the diet composition or to the changes in counterregulatory hormone action (Figure 5E).

It is unclear whether the changes in adipose and liver gene expression cause the change in HOMA-IR or are a result of changes in insulin sensitivity. The changes in GLUT4 gene expression in adipose tissue correlated with loss of regulation of the gluconeogenic gene expression. The possibility of coordinate regulation of GLUT4 in adipose tissue and gluconeogenic gene expression in liver is reinforced by the recent demonstration that down-regulation of GLUT4 in white adipose tissue and up-regulation of gluconeogenic genes are both under control of class II HDAC proteins (42; 43).
While the gluconeogenic gene expression followed predictable patterns for development of hepatic insulin resistance, the changes in lipogenic gene expression were more complicated. Presence of the hGLUT4 transgene altered the expression pattern of SREBP-1c and FAS mRNA under fasted and refeed conditions regardless of the diet regimen (Figure 6A). Normally, SREBP-1c and FAS mRNA is increased in refeed animals as a result of insulin signaling in the liver (41); however, the increased nuclear phosphoCREB (Figure 5E) may be responsible for inhibiting the response to refeeding (44). Presence of the human GLUT4 transgene prevented the normal induction of SREBP-1c and FAS expression, suggesting that normal lipogenesis is altered in the transgenic animals. It is likely that de novo fatty acid synthesis is lower in hGLUT4 TG mice on both CD and HFD diet.

Plasma and hepatic TAG in fasted hGLUT4 TG mice on CD were significantly higher than all other groups. This is most likely due to enhanced lipid release from adipose tissue, and enhanced VLDL secretion from the fasting liver due to the hypoinsulinemia (45). These lipid abnormalities were corrected by HFD in the transgenic mice, reinforcing with notion that relative fasting hypoinsulinemia is the cause of the dyslipidemia.

Diet-induced obesity resulted in a small decrease in GLUT4 expression in brown adipose tissue and skeletal muscle compared to white adipose tissue in both transgenic and non-transgenic mice. It is likely, then, that elevated GLUT4 expression in skeletal muscle and brown adipose tissue are responsible for enhanced insulin sensitivity in the HFD fed hGLUT4 TG mice. In addition to
increased insulin sensitivity, the hGLUT4 transgenic animals on HFD ate 10% more kcal, but had no significant difference in body composition. This suggests that energy output, either through increased activity or uncoupled cellular respiration, was higher in the transgenic mice. The latter explanation is plausible since UCP1 mRNA was lower in NT mice fed HFD compared to transgenic mice (Figure 3).

The reason for enhanced food intake in hGLUT4 transgenic mice fed HFD compared to NT mice is not clear. Regulation of food is complex, and may be related to changes in circulating factors, or could be due to expression of the transgene in GLUT4-expressing neurons. For example, the plasma leptin levels in obese, transgenic mice were significantly lower than the obese NT mice. The decrease in leptin is most likely due to the enhanced insulin sensitivity and generally lower plasma insulin levels in the transgenic mice (46).

In summary, moderate overexpression of GLUT4 is clearly protective against hyperinsulinemia and hyperglycemia in transgenic mice with diet induced obesity. The level of overexpression is similar to the level of overexpression that might be attained by chronic exercise, which gives a direction to pursue for therapeutic intervention. GLUT4 overexpression clearly does not prevent all metabolic adaptations to an obesogenic diet, particularly loss of regulation of hepatic gluconeogenic gene expression. On the other hand, the increased glucose flux through skeletal muscle may limit availability of glucose in liver to be used for de novo fatty acid synthesis. This possibility is predicted by the loss of insulin-dependent SREBP-1c and FAS mRNA expression in the transgenic mice. The
results of this study strongly support that increased expression of GLUT4, particularly in skeletal muscle is a relevant target for treatment of insulin-resistant metabolic disorders.

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B.J.A. researched data and wrote the manuscript. B.A.G., C.D.K. and M.A.J. researched data, A.L.O. researched data, reviewed and edited the manuscript.

A.L.O. is the guarantor of this work, had full access to all of the data, and takes full responsibility for the integrity of the data and the accuracy of the data analysis.

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|                      | NT CD*, n=11 | TG CD, n=11 | NT HFD, n=14 | TG HFD, n=15 |
|----------------------|--------------|-------------|--------------|--------------|
| Fasting Plasma Glucose, mg.dL | Mean (SE)    | Mean (SE)   | Mean (SE)    | Mean (SE)    |
| 0 weeks              | 120 (10)     | 74 (4)     | 130 (7)      | 77 (5)       |
| 4 weeks              | 116 (9)      | 62 (4)     | 171 (10)     | 85 (5)       |
| 8 weeks              | 113 (10)     | 59 (8)     | 178 (11)     | 93 (11)      |
| Fasting Plasma Insulin, ng/mL | Mean (SE)    | Mean (SE)   | Mean (SE)    | Mean (SE)    |
| 0 weeks              | 0.31 (0.04)  | 0.14 (0.03) | 0.41 (0.07)  | 0.20 (0.04)  |
| 4 weeks              | 0.21 (0.03)  | 0.14 (0.03) | 0.45 (0.08)  | 0.17 (0.02)  |
| 8 weeks              | 0.21 (0.02)  | 0.14 (0.02) | 0.59 (0.008) | 0.28 (0.04)  |

*Nontransgenic (NT) and hGLUT4 transgenic (TG) mice fed either control diet (CD) or high fat diet (HFD) for indicated time. Significant differences labeled as follows: ^A indicates p < 0.005 compared to same diet. ^B indicates p < 0.05, compared to same strain. † indicates significant change (p < 0.05) compared to 0 weeks of same group. Analysis for these data was completed using 2-way ANOVA for repeated measurements. There were no interactions between strain and diet.
FIGURE LEGENDS

Figure 1. HOMA-IR scores from NT mice and hGLUT4 TG mice fed the CD or the HFD. HOMA-IR scores were calculated using recorded fasted blood glucose and fasted plasma insulin levels at the time indicated and the following equation: fasting blood glucose (mg/dl) × fasting insulin (µU/ml)/405. The value n= the number of animals used for each analysis. Analysis was completed using 2-way ANOVA using repeated measures. * indicates significant difference compared to week 0 within the same strain. Differences between strains on same diet are indicated on histogram. There were no interactions between strain and diet.

Figure 2. Body composition (A) and Adipose pad weight (B) from NT and hGLUT4 TG mice fed CD or HFD for up to 8 weeks (n=5 to 8 per group). Body composition was measured using a NMR-based method as described in "Materials and Methods". A) Body weight, adipose mass and lean body mass were measured. B) Wet weights for epidydimal white adipose tissue (WAT) and brown adipose tissue (BAT) were reported as a percent of body mass. Data were analyzed by 2-way ANOVA. * indicates a significant difference between diets for the same strain. Differences between strains on same diet are indicated on the histogram.

Figure 3. Average energy intake per week (A) and plasma leptin levels (D) from NT and hGLUT4 TG mice fed CD or HFD (n= 5 to 8 per group). Energy intake (kcal equivalent of diet) was averaged over the 8-week feeding period. * indicates a significant difference between diets. Differences between strains are indicated.
on histogram. Fasting plasma leptin was measured after 0, 4 and 8 weeks on diet. A strain-specific significant difference is indicated. * indicates significant difference between 4 weeks compared to 0 weeks within a strain and diet. # indicates significant difference between 8 weeks compared to 4 weeks within a strain and diet. Data were analyzed by 1-way ANOVA. UCP-1 (B) and UCP-2 (C) mRNA were measured using qRT-PCR and normalized to TATA binding protein (TBP) mRNA for n=4 mice per group. * indicates significant difference between by 1-way ANOVA.

**Figure 4.** GLUT4 protein expression in perigonadal white adipose tissue (A), subscapular brown adipose tissue (C) and hindquarter skeletal muscle (D) and from NT and hGLUT4 TG mice fed CD or HFD for 8 weeks. A representative western blot is shown. Histograms represent quantification of 3 independent experiments. Densitometry is normalized to Akt expression in each tissue. Mouse and transgenic human GLUT4 mRNA expression in perigonadal white adipose tissue (B) is the mean of 3 independent experiments. Analysis for these data was completed using 1-way ANOVA. * indicates difference between diets for the same strain. Differences between strains on same diet are indicated on histogram.

**Figure 5.** Hepatic gluconeogenic gene expression during fasting and refeeding from NT and hGLUT4 TG mice fed CD or HFD for 8 weeks (n=3 to 5 per group). Livers were removed from mice fasted for 17 hours (FASTED) or fasted mice refed for 4 hours (REFED). Hepatic mRNA was isolated and analyzed for
phosphoenolpyruvate carboxy kinase (PEPCK) mRNA (A) and glucose-6-phosphatase mRNA (B). Data were analyzed by 2-way ANOVA. Significant differences between fasted and refed are indicated on histogram. (C) Pyruvate tolerance test for NT and TG mice fed HFD for 8 weeks (n= 5 animals per group). * indicates significant difference between strains at individual time points using 2-tailed student t-test. (D) For in the fasted/refed group, plasma glucose levels were measured before and after refeeding. Plasma insulin was measured after refeeding. Data were analyzed by 2-way ANOVA for repeated measurements. * indicates significant differences for different diets within the same strain. E) Phospho CREB and total CREB immunolabeling of fasted and refed liver nuclear extracts. Histogram represents 3 independent experiments. Data were analyzed by 2-way ANOVA. Differences between fasted and refed animals are indicated on histogram † indicates significant difference compared to all refed animals. * indicates significant between all refed animals compared to NTCD mice, ‡ indicates significant difference between TGFHD and all other fasted mice.

Figure 6. Hepatic lipid metabolism during fasting and refeeding from NT and hGLUT4 TG mice fed CD or HFD for 8 weeks. Livers were removed from mice fasted for 17 hours (FASTED) or fasted mice refed for 4 hours (REFED) after 8 weeks on diet. Hepatic mRNA was isolated and analyzed for SREBP-1c mRNA (A) and Fatty Acid Synthase (FAS) mRNA (4 independent experiments). Lipid soluble material were isolated from blood plasma (n=5 per group) (C) and liver homogenates and assayed for total triacylglycerol (n=5 per group) (D). Statistical
differences between fasted and refed mice are indicated. * indicates significant
difference in refed mice compared between strains and diets. ‡ indicates
significant difference in fasted animals when comparing both strains and diets. #
indicates significant difference in fasted animals when comparing same diet in
different strains. † indicates significant difference in refed animals comparing
same strain.
Figure 1

![Graph showing HOMA IR score over time for different conditions.]

- NTCD (n=7)
- TGCD (n=5)
- NTHFD (n=11)
- TGHFD (n=8)

Comparisons:
- P < 0.01 for certain conditions

Legend:
- 0 week
- 4 weeks
- 8 weeks
Figure 2

A) Comparison of Body Mass, Adipose Mass, and Lean Mass between groups.

- NT CD
- TG CD
- NT HFD
- TG HFD

Significance levels:
- p < 0.01
B) Adipose mass/ body mass, %

- NTCD
- TGCD
- NTHFD
- TGHFD

p < 0.05
Figure 3

A)

Calorie intake (kcal/week)

NTCD  TGCD  NTHFD  TGHFD

p < 0.05

*
Plasma Leptin (ng/mL)

- NTCD
- TGCD
- NTHFD
- TGHFD

- 0 week
- 4 weeks
- 8 weeks

P < 0.01

*  

#
Figure 4

A)

|       | CD   | HFD  |
|-------|------|------|
| NT    | IB: GLUT4 | IB: AKT |
| TG    | IB: GLUT4 | IB: AKT |

p < 0.05

Relative Densitometry (GLUT4/AKT)

- NTCD
- TGCD
- NTHFD
- TGHFD

* Indicates statistical significance at p < 0.05
B) GLUT4 mRNA, relative units

- Mouse GLUT4
- Human GLUT4

Legend:
- NT CD
- TG CD
- NT HFD
- TG HFD

* Indicates significance level.
C) 

Relative Densitometry (GLUT4/AKT)

IB: GLUT4

IB: AKT

p < 0.005
**D)**

![Image of Western Blot](image)

**IB: GLUT4**

**IB: AKT**

|       | CD       | HFD      |
|-------|----------|----------|
|       | NT       | TG       | NT       | TG       |
| **IB: GLUT4** | ![Image] | ![Image] | ![Image] | ![Image] |
| **IB: AKT**    | ![Image] | ![Image] | ![Image] | ![Image] |

- **50 kDa**

**Graph:**

|               | NTCD | TGCD | NTHFD | TGHFD |
|---------------|------|------|-------|-------|
| **Relative Densitometry (GLUT4/AKT)** | ![Bar Graph] | ![Bar Graph] | ![Bar Graph] | ![Bar Graph] |

- **p < 0.005**
- **p < 0.005**
Figure 5

A)

Relative PEPCK mRNA

- **NT CD**
- **TGCD**
- **NTHFD**
- **TGHFD**

- FASTED
- REFEED

*p < 0.01

*p < 0.01

* *
Glucose-6-phosphatase mRNA, relative units

**B)**

- **NT CD**
- **TGCD**
- **NTHFD**
- **TGHFD**

- **FASTED** (open bars)
- **REFED** (filled bars)

**p < 0.01**

* indicates statistical significance.
Fasted blood glucose

Refed blood glucose

Refed plasma insulin

Blood glucose, mg/dL

Serum insulin, ng/mL

D)

NTCD
TGCD
NTHFD
TGHFD

ns

* *
*
*,# *

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E)

Nuclear Extracts

43 kDa → [Image of IB: α-PhosphoCREB]

43 kDa → [Image of IB: α-CREB]

|    | F | R | F | R | F | R | F | R |
|----|---|---|---|---|---|---|---|---|
| NTCD |  |   |   |   |   |   |   |   |
| TGCD |   |   |   |   |   |   |   |   |
| NTHFD |   |   |   |   |   |   |   |   |
| TGHFD |   |   |   |   |   |   |   |   |

p < 0.01
Figure 6

A) SREBP 1c mRNA, relative units

- NT CD
- TGCD
- NTHFD
- TGHFD

FASTED
REFED

p < 0.01

*
B) Fatty Acid Synthase mRNA, relative units

- NT CD
- TG CD
- NT HFD
- TG HFD

p < 0.05
Plasma triacylglycerol, mg/mL

C)  

\[ p < 0.01 \]

\[ ns \]

FASTED  REFED

|       | FASTED | REFED |
|-------|--------|-------|
| NTCD  |        |       |
| TGCD  |        |       |
| NTHFD |        |       |
| THFD  |        |       |
