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Activation of the Hexosamine Signaling Pathway in Adipose Tissue Results in Decreased Serum Adiponectin and Skeletal Muscle Insulin Resistance

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Overexpression of the rate-limiting enzyme for hexosamine synthesis (glutamine:fructose-6-phosphate amidotransferase) in muscle and adipose tissue of transgenic mice was previously shown to result in insulin resistance and hyperleptinemia. Explanted muscle from transgenic mice was not insulin resistant *in vitro*, suggesting that muscle insulin resistance could be mediated by soluble factors from fat tissue. To dissect the relative contributions of muscle and fat to hexosamine-induced insulin resistance, we overexpressed glutamine:fructose-6-phosphate amidotransferase 2.5-fold, specifically in fat under control of the aP2 promoter. Fasting glucose, insulin, and triglycerides were unchanged in the transgenic mice; leptin and β-hydroxybutyrate levels were 91% and 29% higher, respectively. Fasted transgenic mice have mild glucose intolerance and skeletal muscle insulin resistance *in vivo*. In fasting transgenic mice, glucose disposal rates with hyperinsulinemia were decreased 27% in females and 10% in males. Uptake of 2-deoxy-D-glucose into muscle was diminished by 45% in female and 21% in male transgenics. Serum adiponectin was also lower in the fasted transgenics, by 37% in females and 22% in males. TNFα and resistin mRNA levels in adipose tissue were not altered in the fasted transgenics; levels of mRNA for leptin were increased and peroxisome proliferator-activated receptor γ decreased. To further explore the relationship between adiponectin and insulin sensitivity, we examined mice that have been refed for 6 h after a 24-h fast. Refeeding wild-type mice resulted in decreased serum adiponectin and increased leptin. In transgenic mice, however, the regulation of these hormones by refeeding was lost for adiponectin and diminished for leptin. Refed transgenic female and male mice no longer exhibited decreased serum adiponectin in the refed state, and they were no longer insulin resistant as by lower or unchanged insulin and glucose levels. We conclude that increased hexosamine levels in fat, mimicking excess nutrient delivery, are sufficient to cause insulin resistance in skeletal muscle. Changes in serum adiponectin correlate with the insulin resistance of the transgenic animals. (*Endocrinology* 145: 2118–2128, 2004)

ALTHOUGH THERE IS a major genetic contribution to type 2 diabetes, the largest predisposing factor remains caloric excess and/or obesity. Underlining the importance of this mechanism, excess glucose and lipids not only result from but also cause the pathologic hallmarks of diabetes, insulin resistance, and β-cell failure. These feedback cycles make it difficult to dissect the causal relationships that lead to insulin resistance and β-cell failure. A widely accepted viewpoint is that insulin resistance in skeletal muscle is a primary defect that results in compensatory hyperinsulinemia. However, other data suggest that insulin resistance might be largely an acquired defect that is primarily caused by obesity, excess nutrient flux, and/or hyperinsulinemia itself.

One approach to unravel these interactions has been to target specific candidate mechanisms for diabetes to individual organs in transgenic animals. This can potentially allow conclusions to be drawn about necessity and sufficiency of abnormalities in those tissues for the development of diabetes. One hypothesis that has been tested in this fashion is that intracellular nutrient sensing, mediated by products of the hexosamine biosynthesis pathway, may account for the diabetic phenotype. Originally suggested as a pathway that accounted for insulin resistance induced by high concentrations of glucose (1), it now appears that the hexosamine pathway may play a role in other aspects of type 2 diabetes as well, including hyperinsulinemia, hyperlipidemia, hyperleptinemia, and obesity (2–6). Thus, an attractive feature of the hexosamine hypothesis is that it can explain much of type 2 diabetes in terms of caloric excess and normal signaling pathways that operate in parallel in several tissues and participate in the signaling for the partitioning of ingested calories for long-term storage.

One of the hallmarks of type 2 diabetes is resistance to insulin stimulation of glucose uptake in skeletal muscle. We have previously generated transgenic mice that overexpress the rate-limiting enzyme for hexosamine biosynthesis, glutamine:fructose-6-phosphate amidotransferase (GFA) in muscle and adipose tissue under control of the GLUT4 promoter. These mice exhibit total body insulin resistance and decreased uptake of glucose into skeletal muscle (7, 8). We

Abbreviations: Acrp30, Adiponectin (30-kDa adipocyte complement-related protein); 2-DG, 2-deoxy-D-glucose; GFA, glutamine:fructose-6-phosphate amidotransferase; GlcNAc, N-acetylglucosamine; KHB, Krebs-Henseleit bicarbonate buffer; OPA, o-phthaldialdehyde; PPAR, peroxisome proliferator-activated receptor; UCP2, uncoupling protein 2; UDP, uridine diphospho.

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demonstrate here, however, that muscle from these mice is not insulin resistant when explanted and incubated *in vitro*. To test the hypothesis that insulin resistance in these mice is mediated by secretory products of adipose tissue, we therefore generated transgenic mice that overexpress GFA in adipose tissue only. This tissue-specific increase in hexosamine flux, intended to model excess nutrient delivery to fat alone, causes insulin resistance in skeletal muscle. The results support the hypothesis that hexosamines mediate intracellular nutrient/satiety sensing and that skeletal muscle insulin resistance is an autoregulatory response to excess nutrient intake by the organism. The data highlight the important role of the adipocyte and its secreted products in orchestrating these changes in other tissues.

Materials and Methods

Materials

Routine reagents were purchased from Sigma (St. Louis, MO) unless otherwise indicated.

Transgenic mice

The mice overexpressing GFA under control of the GLUT4 promoter have been previously described (7). For overexpression in fat, transgene expression was targeted to adipose tissue using the murine aP2 promoter generously provided by Dr. Bruce Spiegelman (Dana Farber Institute, Boston, MA). The transgene consisted of 5.4 kb of the adipocyte P2 gene promoter (9) linked to the 2046-bp human GFA cDNA (10) and the 398-bp simian virus 40 polyadenylation sequence. The founder animal was a hybrid C57BL6/SJ; for subsequent experiments, the founder and transgenic descendants were bred onto a C57BL6/J background for greater than four generations. Mice were fed a diet based on soy protein, with 10% kcal as fat. They were kept on a 1800–0600 h dark/0600–1800 h light cycle, and 12 h fasting was initiated at 2200, 4 h after initiation of the dark (feeding) period. Other mice used in the RT-PCR studies (8.5 months old, five per category) were subjected to a more prolonged fast (24 h, 0000–0000 h). Tissues were harvested from mice that had been anesthetized with 1.25% avertin and then killed by cervical dislocation. Heterozygous transgenic mice and control wild-type nontransgenic animals from the same litters were used in experiments that were approved by the Laboratory Animal Use Committees at the University of Utah Medical Center and the Salt Lake City Veterans Affairs Medical Center. Data from both male and female animals were pooled for analysis unless otherwise noted. The reported experiments were performed on the offspring of a single founder male. Quantitative PCR was used to estimate copy number of presumably concatamerized transgenes at approximately 5 (not shown).

DNA and RNA analysis

PCR amplification of mouse tail chromosomal DNA was performed to verify the presence of the transgene in offspring of transgenic animals. Mouse tail DNA was extracted and amplified using sense and antisense primers that anneal to nucleotides 376–392 and 1025–1044 of the human GFA coding region, respectively, to yield a 669-bp product (10). These primers span six intron-exon boundaries in the endogenous GFA gene, thus ensuring specificity of the PCR products for the intronless transgene. After denaturation of template at 94 C for 2 min, amplification proceeded through 30 cycles of 94 C for 1 min, 42 C for 1 min, and 72 C for 2 min. PCR products were subjected to electrophoresis in a 1% agarose gel.

The presence of transgene-encoded mRNA was confirmed by RT-PCR followed by restriction digestion at a site unique to the human (transgenic) product. Animals were killed after an overnight fast, and tissues were dissected and frozen in liquid nitrogen. Frozen tissues were pulverized under liquid nitrogen, the powder homogenized in Trizol Reagent, and RNA isolated according to the manufacturer's protocol (Life Technologies, Grand Island, NY). Five micrograms of total RNA were used for first-strand synthesis using Ready-to-Go U-Prime Beads (Amersham Biosciences, Piscataway, NJ) and an oligo deoxythymidine primer. A specific GFA fragment was then amplified using primers hybridizing to nucleotides 89–115 (sense) and 1989–2016 (antisense) of the GFA cDNA. The 1.9-kb product was reamplified using nested primers (sense, 292–320 bp; antisense, 1803–1828 bp) at a 55 C annealing temperature to produce a 1.5-kb fragment. The human sequence but not the mouse sequence contains a unique *Hin*CII site at 1032 bp, with cleavage yielding 739- and 796-bp fragments; any RT-PCR product derived from endogenous mouse GFA mRNA would not be digested by *Hin*CII. Products were analyzed on 1% agarose gels.

Glucose uptake in isolated soleus and extensor digitorum longus muscles

Glucose uptake in isolated muscle was performed as described (11). Dissected muscles were preincubated for 30 min at 35 C in Krebs-Henseleit bicarbonate buffer (KHB) containing 8 mmol/liter glucose, 0.1% BSA, and either 0, 60, or 2000 μU/ml insulin. The muscles were then transferred to KHB without glucose and with 1 mmol/liter pyruvate and the same concentrations of insulin for 10 min at 29 C. Glucose uptake was then quantified by incubating the muscles for 20 min at 29 C in 1 ml of KHB containing 4 mmol/liter 2-deoxy-D-[1,2-^{3}H]glucose (2-DG, 1.5 μCi/ml), and 36 mmol/liter [U-^{14}C]mannitol (0.3 μCi/ml), maintaining the same concentrations of insulin. Extracellular space and intracellular 2-DG were determined as described (12).

Quantitation of mRNA by RT-PCR

Epididymal fat pads from mice, either fasted 24 h or 6 h after being refed, were dissected, placed in 800 μl RNA-Later (Ambion, Austin, TX), and stored at −20 C. Seventy milligrams of the fat pad were placed in 1.4 ml TRI Reagent (MRC, Cincinnati, OH), shredded in a polytron-homogenizer for 10 sec, and further homogenized using a Sonic Dismembrator-60 (Fisher Scientific, Springfield, NJ) at setting 6 for 5 sec. RNA was then prepared according to the manufacturer's protocol and dissolved in 40 μl FORMazol (MRC). RNA concentrations were measured spectrophotometrically. First-strand cDNA synthesis was carried out using 1.3 μg samples of RNA and 300 ng of random hexamer primers (Invitrogen, Carlsbad, CA) in a reaction volume of 25 μl, using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol, with the exception that the final dithiothreitol concentration was reduced to 2 mM (13).

Real-time PCR was performed with a rapid thermal cycler (LightCycler, Roche Diagnostics, Mannheim, Germany) using a modification of a published protocol (14). Reactions (10 μl) were performed using approximately 8 ng cDNA as template with 0.5 μM each primer, 200 μM each deoxynucleotide triphosphate, 50 mM Tris (pH 8.3), 500 μg/ml nonacteylated BSA (Sigma), 3.0 mM $MgCl_2$, 0.04 U/μl Platinum *Taq* DNA polymerase (Invitrogen), and 1:30,000 dilution of SYBR Green I fluorescent dye (Molecular Probes, Eugene, OR). Primers based on murine sequences were chosen using the Primer3 program (15). For resistin, 5′-TTCCTTGTCCCTGAACTGCTG (sense) and 5′-GCTGGAAACCACGCTCACTT (antisense) amplified a 337-bp product. For adiponectin, 5′-GCCAGTCATGCCGAAGA and 5′-TCTCCAGCCCCACACTGAAC amplified a 332-bp product. For aP2, 5′-TGATGCCTTTGTGGGAACCT and 5′-GCTTGTCACCATCTCGTTTTCTCT amplified a 335-bp product. For leptin, 5′-CGGTTCCTGTGGCTTTGG and 5′-GGTCTGAGGCAGGGAGCA amplified a 345-bp product. For TNFα, 5′-GGGCCACCACGCTCTTC and 5′-GGAGTAGACAAGGTACAACCCATC amplified a 291-bp product. For peroxisome proliferator-activated receptor (PPAR) γ, 5′-CCTTGCTGTGGGGATGTCTC and 5′-GCCACCTCTTTGCTCTGCTC amplified a 324-bp product. For uncoupling protein 2 (UCP2), 5′-TGGCAGGTAGCACCACAGG and 5′-CAACAGGGGAGGCGATGA amplified a 345-bp product. For cyclophilin-A, 5′-AGCACTGGAGAGAAAGGATTTGG and 5′-TCTTCTTGCTGGTCTTGCCATT amplified a 349-bp product. Amplification used 26–45 four-step cycles, with the rate of temperature change between steps of 20 C/sec. Steps were 95 C with a 0-sec hold, 60 C with a 0-sec hold, 72 C with an 11-sec hold, and 80 C with a 1-sec hold. Fluorescence was detected during the fourth step at a temperature previously determined to be below the melting temperature of the PCR products. After amplification, a melting curve was generated by slowly heating the double-stranded DNA product. Analyses of the postamplification melting curves and visualization of the DNA products after

agarose gel electrophoresis confirmed the absence of nonspecific DNA products. For each amplification's fluorescence *vs.* cycle line, the LightCycler software determined the second derivative maximum (the threshold cycle at which the fluorescence clearly increased above background). Standard curves of log cDNA *vs.* second derivative maximum (fractional cycle number) were constructed for each quantitated transcript, and for the cyclophilin-A normalization transcript, from cDNA mixes compiled from equal amounts of all 20 fat pad cDNAs. Standard curve points of 0, 2, 4, 8, 12, and 16 ng compiled fat pad cDNA were always included with the same PCR run with the entire set of individual cDNA amplifications of the same transcript. Results for each individual cDNA were normalized by dividing the relative amount of each transcript by the relative amount of cyclophilin-A transcript from the same experiment. Within each experiment the same cocktail mix was used, containing everything but the specific primers.

Assay of GFA activity

Both random-fed wild-type and transgenic mice were killed and tissues were excised. A piece of tissue (~0.2 g) was immediately placed in 0.5 ml of extraction buffer (Complete Mini, Roche Diagnostics). The samples were then minced with micro-scissors and sonicated (Sonic Dismembrator-60, setting 5 for 10 sec at 4 C). The samples were centrifuged at 16,000 × *g* for 20 min at 4 C and the supernatant assayed for GFA activity using a modification of a previously published procedure (16). One hundred twenty microliters of the extract were incubated with 120 μl of reaction mix containing 6 mM fructose-6-phosphate, 12 mM glutamine, 40 mM sodium phosphate (pH 7.4), 1 mM EDTA, and 1 mM dithiothreitol at 37 C for 45 min. The reaction was terminated with 5 μl of 10 M perchloric acid, vortexed, and centrifuged (16,000 × *g*; 4 C) for 15 min. The supernatants (~240 μl) were lipid extracted by adding 400 μl of a 1:4 mixture of tri-n-octylamine: 1,1,2-trichlorofluoroethane, vortexing, and centrifuging at above conditions for 4 min. The aqueous phase (110 μl) was transferred to a 1.5-ml microfuge tube and derivatized with 2 vol o-phthaldialdehyde (OPA) solution (4 mg OPA dissolved in 50 μl ethyl alcohol and added to 5 ml 0.1 M sodium borate and 10 μl 2-mercaptoethanol) for 1 min at room temperature. Samples were then neutralized with 330 μl 0.1 M sodium phosphate (pH 7.4), filtered, and separated over a reverse-phase C18 column (25 cm × 4.6 min Spherisorb ODS, Waters, Milford, MA). Absorbance of the sample eluent was analyzed fluorometrically at 340 nm (excitation) and 460 nm (emission), and peak areas were integrated. OPA-derivatized glucosamine-6-phosphate standards were run separately to determine retention time and to generate a standard curve to correlate area to activity. Activity is expressed as U/mg protein where 1 U represents the generation of 1 pmol glucosamine-6-phosphate/min.

Glucose, insulin, free fatty acid, adiponectin, and triglyceride levels in serum

The following diagnostic kits were used: glucose (glucose oxidase method, Sigma), free fatty acids (acyl-coenzyme A synthetase/acyl coenzyme A oxidase/peroxidase method, Roche Diagnostics), and triglycerides (lipoprotein lipase/glycerol kinase/glycerol phosphate oxidase/peroxidase method, Sigma). Insulin and adiponectin concentrations were measured by using Linco RIA kits (Linco Research Inc., St. Louis, MO).

Uridine diphospho (UDP)-N-acetyl-hexosamines and protein O-glycosylation

Levels of UDP-N-acetyl-hexosamines (consisting of UDP-N-acetyl-glucosamine and UDP-N-acetyl-galactosamine), products of the hexosamine biosynthesis pathway, were measured in fat tissue of wild-type and transgenic mice as described (17). Tissues (~0.2 g) from random-fed wild-type and transgenic mice were homogenized at 4 C in 4 vol perchloric acid (300 mM). The precipitates were centrifuged (10,000 × *g* for 15 min at 4 C), and the lipid was extracted from the supernatants with 2 vol tri-n-octylamine: 1,1,2-trichlorofluoroethane (1:4). The aqueous phase was stored at −80 C until analysis the next day by HPLC. The extracts were filtered (0.45 μm), and HPLC was performed on a Partisil 10.5Ax column (25 cm × 4.6 mm, Waters Corp., Taunton, MA), eluted with a concave gradient from 5 mM potassium phosphate (pH 7.2) to 750 mM potassium phosphate (pH 7.2) over 48 min at flow rate of 1 ml/min. UDP-HexNAc levels were quantified by UV absorption at 254 nm, compared with external standards.

For determination of protein O-linked modification by N-acetylglucosamine (GlcNAc), cell proteins were fractionated by SDS-PAGE and blots stained with a monoclonal antibody to O-linked GlcNAc (RL2, Alexis Biochemicals, Baltimore, MD) as described (18).

Glucose tolerance test

After a 12-h fast, a glucose load of 1 mg/g body weight was administered ip. Tail vein blood was sampled for blood glucose determination (Miles Elite glucometer, Elkhart, IN) from nonsedated animals before and at 5, 15, 30, 60, 90, and 120 min after glucose administration.

Determination of glucose disposal rates

All experiments were performed in weight-matched nonsedated transgenic and littermate wild-type mice using the hyperinsulinemic-euglycemic clamp technique previously described (7). Catheters were implanted into the right internal jugular vein. The animals were allowed to recover from surgery for 3 d and then fasted 12 h before the experiment. Animals were infused with recombinant human insulin (HumulinR, Eli Lilly & Co., Indianapolis, IN) at a rate of 20 mU/kg·min, whereas 50% dextrose was infused by a variable infusion pump (Harvard Apparatus Inc., South Natick, MA). Whole blood samples (3 μl) were collected every 5–10 min from tail bleeds and measured by glucometer.

2-DG uptake into skeletal muscle in vivo

After serum glucose levels were stabilized under euglycemic clamp conditions for approximately 60 min, a bolus injection of 2-DG (200 pmol, 11 Ci/mmol; Amersham Biosciences) was administered. Clamp conditions were continued for 10 min after which the animal was killed, blood collected, and the triceps surae muscle group removed and immediately frozen in liquid nitrogen. The triceps surae group was selected because its muscle fiber composition is representative of total hind limb fiber composition (19). The muscle was processed as described (20). Weighed muscle was dissolved in 0.5 ml of 1 M NaOH and incubated in a shaking water bath for 1 h at 60 C. Samples were neutralized with 0.5 ml of 1 M HCl and centrifuged for 10 min. Two 0.5-ml aliquots of the supernatant were collected. One aliquot was deproteinized with 250 μl saturated $Ba(OH)_2$ and 250 μl of $ZnSO_4$ (5.5%), and the second with 0.5 ml $HClO_4$ (6.0%). Both were centrifuged 10 min and the supernatant collected. The $HClO_4$ aliquot contains both phosphorylated and unphosphorylated 2-DG, whereas the $Ba(OH)_2$ and $ZnSO_4$ aliquot contains only the unphosphorylated form. The difference in disintegrations per minute between the two supernatants represent the muscle content of 2-DG-phosphate.

Results

Glucose uptake in isolated skeletal muscle from transgenic mice overexpressing GFA in skeletal muscle and fat under control of the GLUT4 promoter

We have previously demonstrated that mice overexpressing the rate limiting enzyme for hexosamine synthesis, GFA, under control of the GLUT4 promoter are insulin resistant (7). These GLUT4-GFA mice have decreased whole-body glucose disposal rates under conditions of hyperinsulinemia that are paralleled by decreased uptake *in vivo* of 2-DG into hind limb skeletal muscle (7, 8). Because of more recent data demonstrating the regulation of insulin sensitivity by adipocyte-derived factors, we sought to determine whether the insulin resistance of isolated skeletal muscle from these GLUT4-GFA mice normalized after explantation, that is, after removal from the influence of adipose tissue. Neither soleus nor extensor digitorum longus muscles from GLUT4-

GFA were insulin resistant when explanted and exposed to insulin *in vitro* (Fig. 1). Both the magnitude of and the insulin dose responsiveness for 2-DG uptake were equal in muscles from transgenic mice and their littermate controls.

Fat-specific overexpression of GFA in transgenic mice

The previous results suggest that most of the insulin resistance resulting from GFA overexpression under control of the GLUT4 promoter was mediated by GFA overexpression in fat rather than skeletal muscle. To test this hypothesis directly, a transgene was constructed with GFA under control of the aP2 promoter. Expression of this aP2-GFA transgene in the offspring of a single founder male was initially demonstrated by RT-PCR using mRNA from fat. The product was determined to be from the transgene's human GFA sequence based on the presence of a restriction enzyme site in the resulting PCR fragment that is unique to the human GFA sequence (not shown). The direct demonstration of overexpression of the transgene product was made by assaying GFA enzyme activity *in vitro* in several tissues including fat. GFA activity in transgenic fat is approximately 2.5-fold greater than in wild-type fat (Fig. 2A). This increase in GFA activity was paralleled by a 40% increase in fat of the levels of the end product of the hexosamine pathway, UDP-N-acetyl glucosamine (UDP-GlcNAc, Fig. 2B). GFA activity in brain, muscle, kidney, and liver, and UDP-GlcNAc levels in kidney were not different in transgenics compared with wild types. There is a slight but not significant increase in GFA activity measured in muscle. Histologic analysis of muscle revealed increased interstitial adipose tissue in the transgenic animals (not shown), and this may contribute disproportionately to the observed levels of GFA in whole muscle because GFA activity is significantly higher in fat *vs.* muscle (Fig. 2A). Other measures of hexosamine flux in muscle revealed no differences between control and transgenic mice (Table 2).

Although the total intracellular levels of GFA and UDP-GlcNAc were only modestly elevated in the transgenic fat, there was a relatively larger difference in the level of O-linked-GlcNAc on cytosolic proteins in fat from transgenic mice (Fig. 2C) as determined by Western blotting with an antibody specific for that modification. Levels of O-linked GlcNAc are known to be substrate limited by UDP-GlcNAc (21).

General characteristics of the transgenic animals

The transgenic mice showed no overt abnormalities compared with their littermate wild-type controls, and their longevity is comparable. As shown in Table 1, weights, fasting glucose, insulin, free fatty acid, and triglyceride levels were comparable in 3-month-old wild-type and transgenic animals. A trend toward higher fasting insulin and glucose levels in the transgenic animals was not statistically significant. Leptin levels were significantly increased in fasted transgenics, consistent with previous reports that hexosamines increase leptin synthesis (4, 22, 23). Leptin levels increase significantly with refeeding in both transgenic and wild-type control mice, reaching a plateau that is not different in transgenics compared with wild types, as has also been observed in mice overexpressing GFA in muscle and fat (23). There was a modest (29%) but significant ($P < 0.05$) increase of fasting β-hydroxybutyrate in the transgenic mice.

Transgenic male mice are glucose intolerant

Glucose tolerance was assessed in 3-month-old male and female mice after ip injection of 1 mg glucose/g body weight. Female animals had normal glucose tolerance (Fig. 3A), whereas males had impaired glucose tolerance (Fig. 3B). The glucose values at 60 and 120 min were significantly higher in the males, as was the integrated area under the glucose curve (Fig. 3C).

Transgenic animals have insulin resistance and decreased 2-DG uptake into skeletal muscle in vivo

The finding of decreased glucose tolerance (Fig. 3C) without lower insulin levels (Table 1) in the transgenic mice suggests that they are insulin resistant. We therefore examined total body glucose disposal rates and glucose uptake into muscle tissue *in vivo*. Both male and female transgenic animals were insulin resistant for total body glucose uptake as determined by the hyperinsulinemic-euglycemic clamp technique. Glucose disposal rates in transgenic females were 27% lower than in wild-type littermates (Fig. 4A, $P < 0.0005$). Importantly, these studies were performed in young female mice with weights (Table 1) and glucose tolerance (Fig. 3A) identical between transgenic and wild type. Body composition analysis of these young females also showed identical body fat content (wild type, 9.8 ± 0.9% body fat; transgenic, 9.7 ± 0.8% body fat, n = 5 each). Thus, the marked insulin resistance is not explained as a secondary effect of adiposity or glucose intolerance. Male transgenic mice were also insulin resistant compared with male controls (Fig. 4B, $P = 0.02$). The magnitude of the decrease in glucose disposal rates of the trangenics was 10%, less than that observed for the females.

To demonstrate directly that glucose uptake into muscle

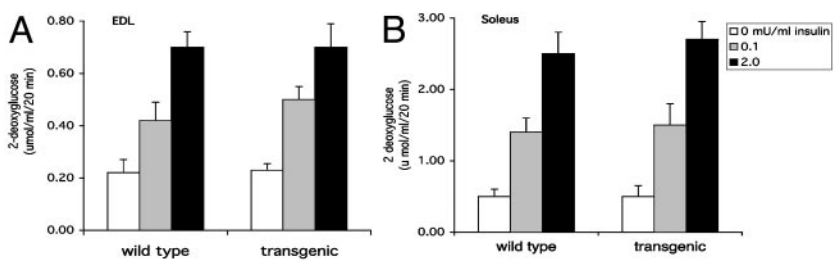

FIG. 1. Lack of insulin resistance in explanted muscles from insulin-resistant mice overexpressing GFA in muscle and fat under control of the GLUT4 promoter. 2-DG uptake was determined in soleus (A) and extensor digitorum longus (EDL) (B) muscles from transgenic mice and their nontransgenic littermates. Results are the means (±SE) of eight to 10 independent determinations for each muscle at each concentration of insulin. The transgenic mice did not differ from wild types in their total body weights or in the weights of the isolated muscles (not shown).

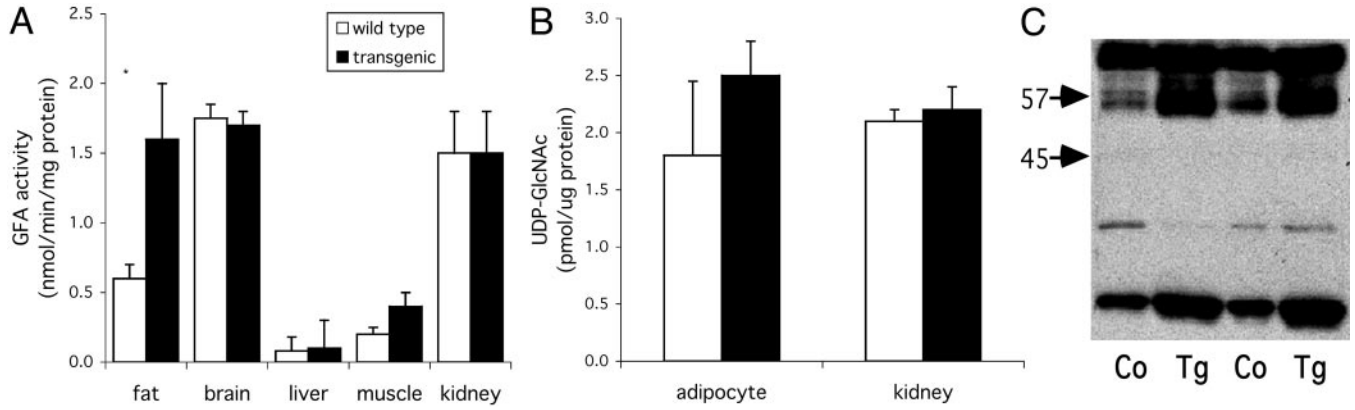

FIG. 2. GFA overexpression targeted to adipose tissue in aP2-GFA transgenic mice. A, GFA activity was assayed in tissues from wild-type (wt) and transgenic (Tg) mice that had been fasted 12 h. (n = 5–7 per group, $P < 0.02$ for activity in wt *vs.* Tg fat; all others, P = NS). B, UDP-N-acetyl glucosamine levels in isolated adipocytes (pmol/mg fat pad) or in kidney tissue (pmol/μg protein) in wt and Tg mice (n = 5 each fat pads and n = 7 each kidney samples, each sample from an individual mouse). C, Levels of O-GlcNAc-modified proteins were determined by Western blotting of protein extracts from control (Co) and Tg fat pads using an antibody (RL2) that recognizes the O-GlcNAc moiety. Each lane was normalized for protein and was derived from a fat pad from an individual mouse.

TABLE 1. Weights and serum chemistries in transgenic and wild-type mice

		Wild type	Transgenic
Weight (g)	Male:	25.5 ± 0.3	26.2 ± 0.4
	Female:	20.8 ± 0.5	20.8 ± 0.4
Fasting glucose (mM)	Male:	4.59 ± 0.58	5.00 ± 0.31
	Female:	4.78 ± 0.38	5.18 ± 0.46
Fasting insulin (pM)		60.7 ± 1.1	62.3 ± 1.5
Fasting leptin (ng/ml)		1.1 ± 0.1	2.1 ± 0.3[a]
Refed leptin (ng/ml)		5.9 ± 0.4[b]	5.3 ± 0.3[b]
Fasting free fatty acids (mM)		0.42 ± 0.04	0.42 ± 0.08
Fasting β hydroxybutyrate (mg/dl)		8.0 ± 1.0	10.3 ± 1.9[a]
Fasting triglycerides (mg/dl)		43.7 ± 4.2	45.2 ± 3.3

All values are the means ± SE of seven to 20 determinations each, wild-type and transgenic animals, all 2–3 months of age. Other than weight and glucose, there were no differences between the sexes and values were pooled for males and females in those cases.

[a] $P < 0.05$, wild-type *vs.* transgenic by *t* test. [b] $P < 0.001$, refed *vs.* fasted animals.

was impaired in these animals, mice were infused with 2-DG at the end of the euglycemic clamp studies. Hind limb muscle was then excised, carefully dissected free of fat, and radioisotope uptake determined. Uptake of 2-DG was decreased by 45% in muscle of the female aP2-GFA transgenic mice, paralleling the decrease of total body glucose disposal seen in the euglycemic clamp study (Fig. 4C). Uptake of 2-DG into muscles of male transgenic mice was 21% lower than controls (Fig. 4D).

We also determined that the observed insulin resistance was not due to increased levels of triglyceride, hexosamine flux, or protein O-glycosylation within skeletal muscle (Table 2).

Muscle insulin resistance of aP2-GFA muscle is lost after explantation

The findings of Fig. 1 showed loss of insulin resistance after explanting muscles from the GLUT4-GFA mice (with fat and muscle overexpression of GFA) and analyzing their glucose uptake *in vitro*. We therefore sought to replicate that finding in the fat-specific aP2 model. Muscles from these transgenic mice were not insulin resistant *in vitro* (Fig. 5), demonstrating again that the insulin-resistant phenotype in muscle is dependent on extrinsic factors.

Insulin resistance is associated with decreased serum adiponectin (Acrp30) in fasted transgenic mice

We next examined possible mediators of insulin resistance in the transgenic animals. Adiponectin is a secreted product of the adipocyte whose levels have been shown to be inversely related to insulin resistance, obesity, and diabetes (24–27). Because the glucose tolerance tests and the hyperinsulinemic clamps were performed in fasting animals, we first examined serum adiponectin levels after fasting. Serum adiponectin levels were significantly lower in the fasted transgenic animals, both female and male, compared with wild types (Fig. 6, $P < 0.05$). Levels of adiponectin were higher in females than in males, as has been previously reported (28).

RT-PCR quantification of other mRNAs possibly involved in insulin resistance

Levels of mRNA for several other candidate mediators of insulin resistance were next examined in fasted transgenic and wild-type mice (Fig. 7). We first verified overexpression of GFA in epididymal fat pads of 3- to 8-month-old, 24-h-fasted transgenic animals, using primers that recognize both mouse and human GFA; total GFA levels were increased 2.2-fold in transgenic fat ($P < 0.05$), in good agreement with the increase in enzyme activity presented in Fig. 2. We also verified that the use of the aP2 promoter for expression of the transgene did not interfere with the expression of endogenous aP2.

We previously reported that leptin is up-regulated by increased hexosamine flux in transgenic mice overexpressing GFA under control of the GLUT4 promoter (23). Serum leptin (Table 1) and leptin mRNA (Fig. 7, $P < 0.05$) are both increased in fasting aP2-GFA transgenic mice. Two other candidates for causing insulin resistance in muscle, resistin (29), and TNFα (30) did not differ in mRNA levels between wild-

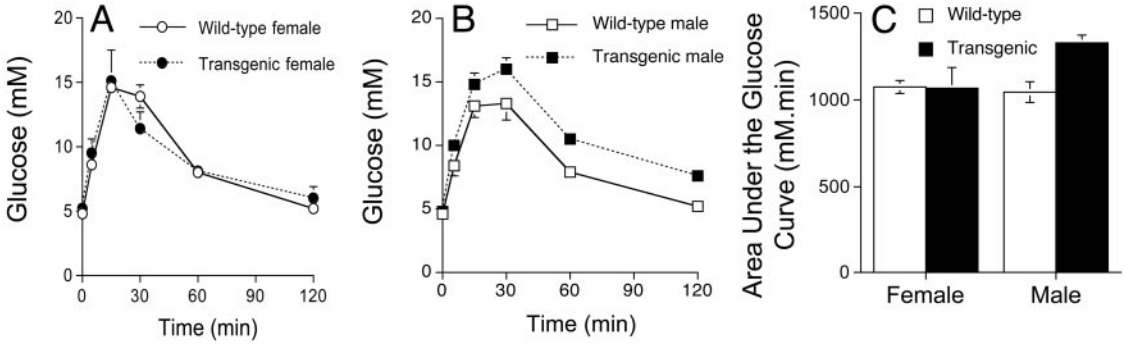

FIG. 3. Glucose tolerance as assessed by ip glucose challenge. Female (A) and male (B) mice were given 1 mg glucose/g body weight ip. Glucose levels were monitored by tail vein at the indicated times. Transgenic animals (n = 3 female and 7 male) were compared with their littermate wild-type controls (n = 5 female and 3 male). Animals were of equal weight. C, The areas under the glucose curves for the individual animals were integrated. Results are the means ± SE. *, $P < 0.05$, comparing the means of wild-type to transgenic mice by t test.

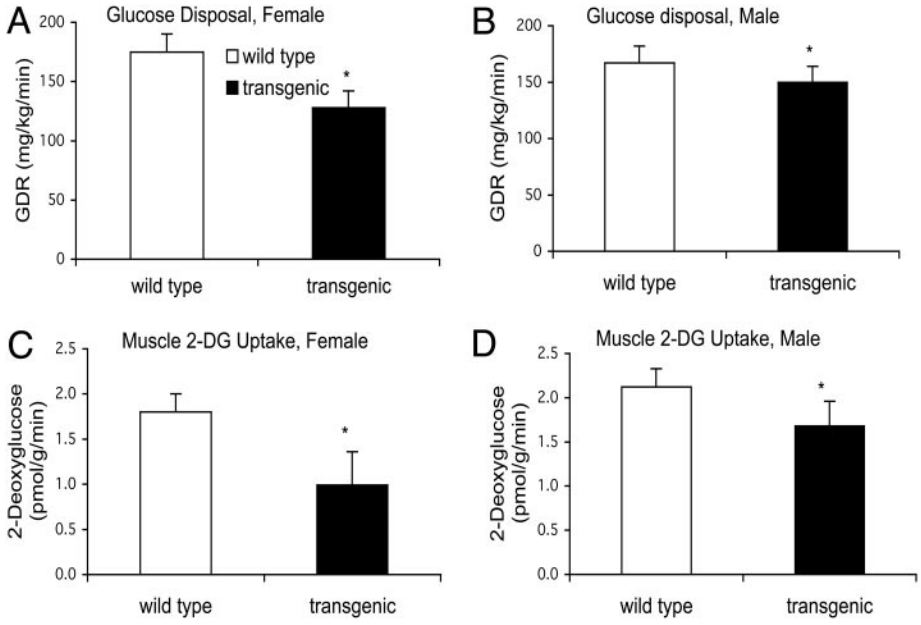

FIG. 4. Transgenic aP2-GFA mice are insulin resistant for total body and skeletal muscle glucose uptake. Glucose disposal rates were determined by the hyperinsulinemic-euglycemic clamp technique in female (A) and male (B) mice. Results are the means (±SE) of 11–14 mice per group. Steady-state glucose levels during the clamp were comparable in the two groups (wild type, 7.0 ± 1.5; transgenic, 7.7 ± 1.5 mM). Glucose uptake into skeletal muscle was determined in female (C) and male (D) mice at the end of the clamp by infusing a bolus of 2-DG and measuring 2-DG-phosphate in muscle extracts (n = 4–6 mice per group, each assayed in two isolated pieces of muscle tissue). Differences between the wild-type and transgenic animals are significant ($P < 0.05$) for both glucose disposal rates and 2-DG uptake.

TABLE 2. Muscle triglyceride and hexosamine levels

	Wild type	Transgenic
Muscle triglyceride (mg/μg protein)	0.25 ± 0.03	0.23 ± 0.03
Protein O-GlcNAc (arbitrary density)	9.9 ± 1.2	8.1 ± 0.8
UDP-GlcNAc (nmol/μg protein)	60.7 ± 1.1	62.3 ± 1.5

Values are the means ± SE of four to 11 determinations from individual wild-type and transgenic animals. The values shown are from fasted males and females, but the values did not vary between the sexes nor with fasting/refeeding (not shown).

type and transgenic mice (Fig. 7), nor did the corresponding serum levels (not shown). Levels of PPARγ were decreased 30% in the fasted transgenic mice compared with fasted wild types ($P < 0.005$). UCP2 was not significantly decreased (16%, $P = 0.28$). Adiponectin mRNA was decreased, although this difference was not statistically significant (10% decrease, $P = 0.12$).

Dysregulation of adiponectin after fasting/refeeding in transgenic mice

The results in Figs. 4 and 6 demonstrate that insulin resistance and decreased serum adiponectin levels occur in fasted transgenic animals. We also examined mice 6 h after refeeding subsequent to a prolonged fast of 24 h. Serum adiponectin fell by 43% in female wild-type mice after refeeding (Figs. 6 and 8A, $P = 0.03$) and by 39% in wild-type males after refeeding ($P = 0.04$). These decreases in serum adiponectin levels were paralleled by decreases in adiponectin mRNA in the refed mice ($P < 0.001$, data not shown). Unlike the wild-type mice, however, serum adiponectin levels did not decrease in the transgenic mice after refeeding. The net result is that the refed transgenic mice no longer exhibited lower serum adiponectin levels compared with wild types.

Increased insulin sensitivity associated with increased adiponectin levels in refed transgenic mice

In the fasted transgenic mice, the decreased level of adiponectin was associated with insulin resistance. In the refed transgenic mice, however, this difference in serum adiponectin levels was lost (Fig. 8A). If adiponectin is responsible for the insulin resistance seen in the fasted transgenic mice (Figs. 3 and 4), then it would be predicted that transgenic mice would not be insulin resistant compared with wild types in

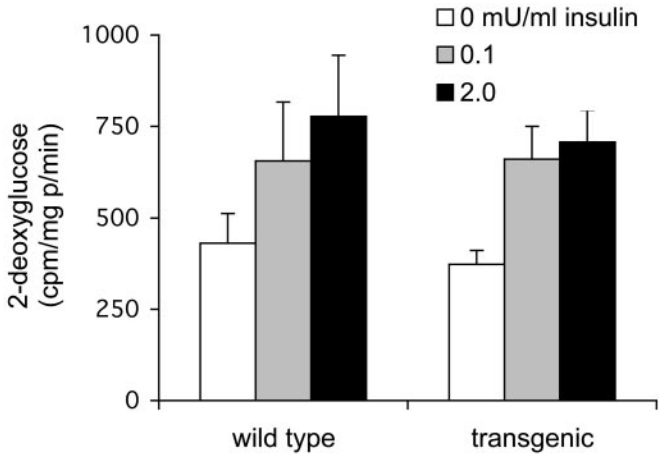

FIG. 5. Lack of insulin resistance in muscles explanted from mice overexpressing GFA in fat under control of the aP2 promoter. 2-DG uptake was determined in soleus muscles from transgenic mice and their nontransgenic littermates. Results are the means (±SE) of six to eight independent determinations for each muscle at each concentration of insulin. The transgenic mice did not differ from wild types in their total body weights or in the weights of the isolated muscles (not shown).

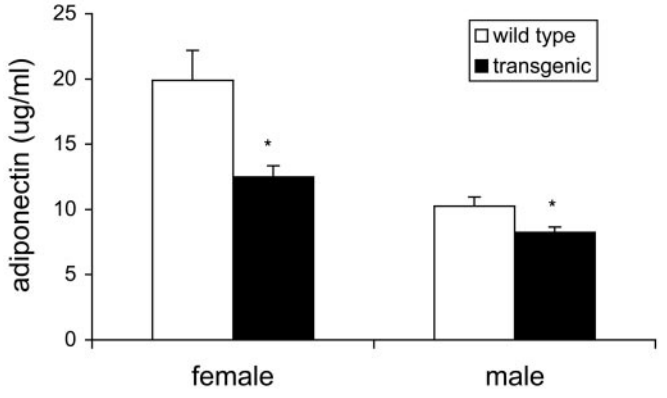

FIG. 6. Serum adiponectin levels are decreased in transgenic mice. Adiponectin was measured in serum from fasted male and female transgenic and wild-type mice. Results are the mean ± SE for four to six animals per group. *, $P < 0.05$ for transgenic compared with wild-type mice.

the refed state. This was the case (Fig. 8, B–D). Insulin sensitivity was assessed by measuring serum insulin and glucose levels after a 24 h fast followed by a 6-h period of refeeding. Refed transgenic females had significantly lower insulin levels than wild types ($P = 0.02$) despite similar glucose levels (P = NS). The product of insulin multiplied by glucose was also significantly lower in transgenic females ($P < 0.02$). The same trend was seen in males, although the differences were not statistically significant.

Discussion

We have earlier reported that mice overexpressing GFA in fat and muscle were insulin resistant for glucose uptake into skeletal muscle *in vivo* (7, 8). Here we demonstrate that the insulin resistance is reversed upon explanting those muscles and stimulating with insulin *in vitro*. Furthermore, we demonstrate that fat-specific overexpression of GFA, the rate-limiting enzyme for hexosamine synthesis, is sufficient to cause insulin resistance in skeletal muscle. These results further confirm the role of the hexosamine pathway in nutrient sensing and insulin resistance (2, 3). The findings also support an active role for fat tissue in controlling total body fuel homeostasis. Similar conclusions were reached using mice wherein adipose-specific disruption of the GLUT4 gene led to insulin resistance *in vivo* in muscle and liver, whereas insulin resistance was not seen in explanted muscle (31). The results support the hypothesis that nutrient-induced insulin resistance in muscle can be a response to the delivery of excess calories to the organism, or more specifically, to adipose tissue. We and others (2, 3, 32) have argued that insulin resistance in muscle is a physiologic and adaptive response to excess calories that autoregulates muscle glucose uptake and favors shunting of excess calories to fat. This conclusion is further strengthened by the response of the adipocytes themselves to increased hexosamine flux, namely insulin resistance but net up-regulation of glucose uptake into fat mediated by up-regulation of GLUT4 (our manuscript in preparation).

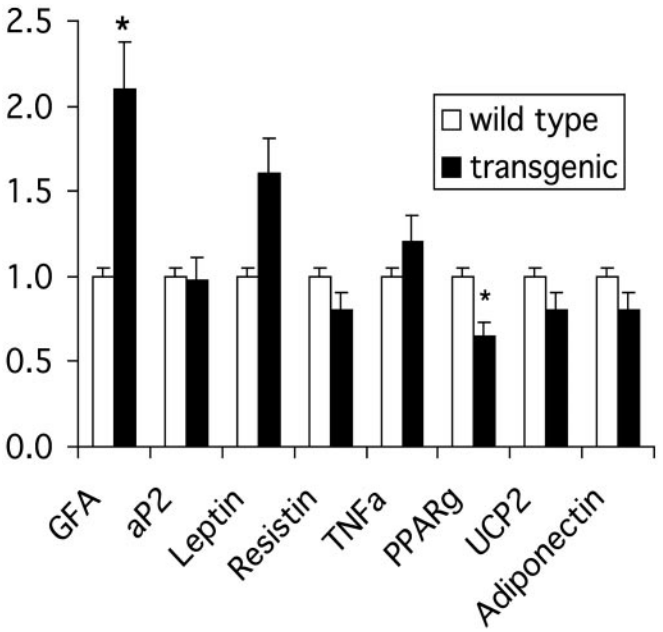

FIG. 7. Levels of mRNA for GFA, aP2, leptin, resistin, TNFα, PPARγ, UCP2, and adiponectin in fat cells overexpressing GFA. Total RNA was purified from epididymal fat pads of wild-type and aP2-GFA mice after fasting. Results for each cDNA were normalized to the levels of cyclophilin A cDNA, and the average level of each mRNA in the fasted wild-type mice was set at unity. Results are the means (±SE) of determinations from five separate fat pads, each from a different mouse, with each assayed in quadruplicate (UCP2 and adiponectin), triplicate (PPARγ and leptin), duplicate (GFA, aP2, and TNFα) or once (resistin). Mice were fasted for 24 h before harvesting tissue. *, $P < 0.05$ for transgenic compared with wild-type mice.

We were able to generate only one line of transgenic animals for the current study. However, the reported results are most likely specific for GFA overexpression and not, for example, due to a nonspecific gene insertion event. First, the animals exhibit a phenotype that is consistent with that observed when GFA is overexpressed in other tissues. In particular, we previously observed insulin resistance and hy-

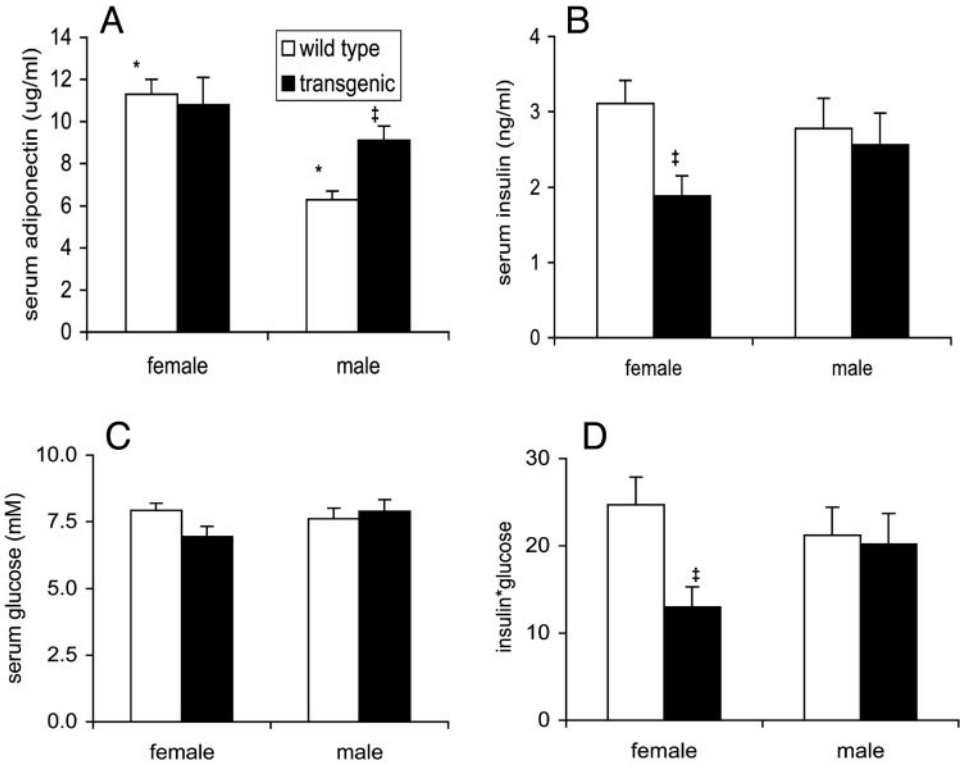

FIG. 8. Serum adiponectin, insulin, glucose, and the product of insulin multiplied by glucose in refed male and female mice. Mice were fasted for 24 h followed by a 6-h period of refeeding. Adiponectin (A), glucose (B), and insulin (C) were measured in tail vein blood. The product of insulin and glucose were calculated as an indirect measure of insulin sensitivity (D). n = 4 female controls, 9 female transgenics, 10 male wild-type controls, 7 male transgenics. *, $P < 0.05$ for refed compared with fasted mice (Fig. 6); ‡, $P < 0.05$ for transgenic compared with wild-type mice.

perleptinemia with GFA overexpression in muscle plus fat under control of the GLUT4 promoter (7, 8). The phenotype of the aP2-GFA mice is also not attributable to interference with endogenous aP2 transcription as demonstrated by the lack of a difference in aP2 mRNA levels in wild-type mice compared with transgenic mice. It should be pointed out that the aP2 promoter is not completely adipose specific in its targeting of gene expression: macrophages express proteins under the control of the aP2 promoter as well (33). Although macrophages produce cytokines that could cause insulin resistance, this is unlikely to be the explanation for the insulin resistance observed in our transgenic animals because primary cultures of their adipocytes are insulin resistant *in vitro* (our manuscript in preparation). The levels of increased hexosamine activity in the transgenic animals are within the range seen in hyperglycemic states and high-calorie feeding. Thus, the results are likely to mirror physiologic regulation by hexosamine flux.

Our findings do not rule out a direct role for hexosamines in the regulation of muscle cell metabolism. In fact, direct exposure of isolated muscle or cultured myocytes to glucosamine does result in insulin resistance in those cells (34, 35). Thus, it is possible that hexosamine flux may induce insulin resistance due to mechanisms that are both adipose specific as well as intrinsic to the myocyte. The current studies demonstrate only that the adipose-specific mechanisms are sufficient and perhaps more sensitive to nutrient flux than those in the myocyte. Whether these putative dual signals for insulin resistance are additive or simply redundant is not known. When GFA is overexpressed in muscle plus fat, the maximal glucose disposal rates were decreased 47% compared with wild types (7). In the current study, using animals with the same genetic background (C57BL6/J), overexpression of GFA in fat led to a 10% and 34% decrease in glucose disposal in males and females, respectively. This smaller decrement in glucose disposal seen in the mice overexpressing GFA only in fat is consistent with the possibility of a direct contribution of myocyte hexosamine flux to insulin resistance. It is possible that this small degree of insulin resistance intrinsic to the muscle itself would not have been detected in the explanted muscle strips.

The mechanisms by which delivery of excess nutrients to fat lead to insulin resistance in muscle are not known at this time. Fat might signal muscle by either nutrient or paracrine/endocrine mechanisms. Fatty acids can induce insulin resistance in muscle, and myocytes could be exposed to free fatty acids liberated locally by lipoprotein lipase. Consistent with this possibility, we do observe increased numbers of interstitial fat cells in transgenic muscle tissue (not shown). However, we did not see systemic increases in serum levels of fatty acids or triglycerides. Levels of intramyocellular triglycerides and hexosamine products were also not increased in the transgenic animals, suggesting that they are not becoming insulin resistant due to changes in muscle hexosamine levels. Fatty acid oxidation was probably increased in the transgenic animals, accounting for the increased levels of β-hydroxybutyrate.

The other plausible mechanism for fat-induced insulin resistance would be an endocrine one. Fat produces hormones and cytokines that can affect insulin sensitivity in muscle, including TNFα (30), resistin (29), leptin (36) and adiponectin (also referred to as Acrp30) (24–27, 37). Neither TNFα nor resistin were altered in the transgenic mice in such a way that might explain the observed insulin resistance.

Leptin mRNA was increased in the fat pads of the aP2-GFA transgenic mice, as were serum leptin levels, consistent with previous observations on the effects of hexosamine flux on leptin synthesis (4, 22, 23). Leptin, however, has been reported to increase insulin sensitivity, so it is not likely to mediate the insulin resistance in the aP2-GFA mice (38–41).

A more attractive candidate for hexosamine-mediated insulin resistance is the molecule adiponectin. Decreased adiponectin levels have been implicated in causing insulin resistance (24–26, 37). In the GFA overexpression model, we have found that the hexosamine pathway does affect adiponectin regulation. Serum adiponectin is decreased in the transgenic mice, and these mice are insulin resistant when assessed by the hyperinsulinemic euglycemic clamp technique and by glucose tolerance testing, both of which are performed in the fasted state. However, the current studies also demonstrate that fasting and refeeding regulate adiponectin in wild-type animals. After refeeding, a decrease in adiponectin is seen in wild-type mice. This normal regulation of adiponectin is lost in the transgenic animals, however. The net result is that unlike in the fasted state, adiponectin levels in refed transgenic mice are the same or higher than in wild types. One would therefore predict no insulin resistance and perhaps even increased insulin sensitivity in refed transgenic mice. This was the case. In refed transgenic females, insulin levels were lower despite similar glucose levels. The product of insulin times glucose, an indirect surrogate measure of insulin sensitivity, was also lower in females. In males, there was a smaller, nonsignificant decrease in these same measures. Thus, the refed transgenic animals are clearly not insulin resistant and the females, at least, exhibit increased insulin sensitivity.

The basis for the regulation of adiponectin by feeding and hexosamines is not known. One previous study has demonstrated decreased adiponectin gene expression with 48 h of fasting compared with 3 d of refeeding in rats (42), the opposite of what is seen in our wild-type C57BL6 mice in a very different fasting/refeeding protocol. However, another study has demonstrated increased adiponectin with calorie restriction (28). The responses of adiponectin and PPARγ to hexosamine flux may be interrelated because PPARγ is a known regulator of adiponectin and is down-regulated in the aP2-GFA transgenic mice (43). PPARγ might also play a more direct role within the adipocyte as a mediator of hexosamine-induced insulin resistance. Although causative roles for adiponectin and PPARγ have been demonstrated in other models of insulin resistance, the current studies do not provide direct evidence for which, if either, are directly responsible for the observed insulin resistance or whether their levels of expression are interrelated. The fact that adiponectin is not the only determinant of insulin sensitivity is clearly shown by the sexual dimorphisms revealed in these studies. Within the sexes, serum adiponectin does correlate with insulin sensitivity, but glucose disposal rates in fasting males in females are very similar despite dissimilar adiponectin levels.

Current data suggest that hexosamines regulate metabolism through the mechanism of O-linked glycosylation of cytosolic proteins. The levels of the end product of the hexosamine pathway, UDP-N-acetylglucosamine, are limiting for modification of proteins by cytosolic O-glycosyl transferase (21, 44, 45). Modification of proteins by the addition of serine- or threonine-linked N-acetylglucosamine is widespread, dynamic, and highly regulated, occurring for example on many transcription factors, cytoskeletal proteins, and nuclear pore proteins (45). The degrees of this modification are responsive to changes in extracellular glucose concentrations and hexosamine flux (46) and therefore could theoretically serve a nutrient sensing function. Furthermore, the proteins homologous to O-glycosyl transferase in *arabidopsis* (SPINDLY) and *Saccharomyces cerevisiae* (*ssn6*) are involved in nutrient sensing (47). Direct evidence for the role of O-glycosylation in metabolic regulation has recently been presented. Pharmacologic inhibition of the enzyme responsible for removal of O-linked GlcNAc, leading to increased levels of O-glycosylation, results in insulin resistance in cultured adipocytes (48). Transgenic overexpression of O-glycosyl transferase under control of the GLUT4 promoter also leads to insulin resistance and hyperleptinemia, mimicking the phenotype seen when GFA is overexpressed with the same promoter (49). Finally, modification of glycogen synthase by O-GlcNAc has been shown to directly result in inactivation and insulin resistance of that enzyme (50).

How, in turn, the O-GlcNAc modification regulates protein activity is beginning to be understood. There is evidence to suggest that O-linked glycosylation affects proteasomal degradation and transcriptional activity of the transcription factor Sp1 (51–53). Sp1, in turn, has been implicated in the transcriptional regulation of a number of glucose- and hexosamine-regulated proteins such as TGFα, TGFβ, and plasminogen activator inibitor-1 (54–56). Several other proteins of direct relevance to diabetes are also regulated by hexosamines, including the transcription factor sterol regulatory element-binding protein 1 (57), glycogen synthase (50), endothelial nitric oxide synthase (58), and several transcription factors (45). The O-glycosyl transferase has also been recently shown to stimulate the recruitment of nuclear proteins into corepressor complexes (59). The levels of O-linked GlcNAc protein modification were increased in the aP2-GFA transgenic fat pads, although the identity and the functional significance of the modified proteins are not yet established.

The current data add to a growing body of evidence that hexosamine flux is used by tissues to sense the nutrient status of the organism to coordinate changes in cell growth and metabolism. The pattern of changes seen with overexpression of GFA in several tissues—hyperinsulinemia, muscle insulin resistance, increased glycogen, and fat synthesis in the liver—can be seen as an adaptive response of cells to direct excess calories to storage as fat. However, these same pathways can also have detrimental consequences, especially when chronically stimulated, including insulin resistance, obesity, hyperlipidemia, β-cell failure, and type 2 diabetes. A longitudinal study of the mice with overexpression of GFA targeted to fat indicates that these mice do develop glucose intolerance and gain excess weight as they age (our manuscript in preparation). Use of these and other models of increased hexosamine flux should aid in understanding the mechanisms underlying these detrimental consequences of chronic overnutrition and their link to type 2 diabetes. The current studies also point to a direct role of the adipocyte and

its secreted products, particularly adiponectin, in these processes.

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