

Resistance to diet-induced obesity and improved insulin sensitivity in mice with an RGS insensitive G184S Gnai2 allele

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Running title: Metabolic alterations in $G\alpha_{i2}^{G184S}$ mice

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Abstract

Objective: Guanine nucleotide binding protein (G protein) mediated signaling plays major roles in endocrine/metabolic function. Regulators of G protein signaling (RGS proteins) are responsible for the subsecond turn-off of G protein signaling and are inhibitors of signal transduction *in vitro*, but the physiological function of RGS proteins remains poorly defined in part due to functional redundancy.

Research Design and Methods: We explore the role of RGS proteins and $G\alpha_{i2}$ in the physiologic regulation of body weight and glucose homeostasis by studying genomic “knock-in” mice expressing RGS-insensitive $G\alpha_{i2}$ with a G184S mutation that blocks RGS protein binding and GTPase acceleration.

Results: Homozygous $G\alpha_{i2}^{G184S}$ knock-in mice show slightly reduced adiposity. On a high-fat diet, male $G\alpha_{i2}^{G184S}$ mice are resistant to weight gain, have decreased body fat, and are protected from insulin resistance. This appears to be a result of increased energy expenditure. Both male and female $G\alpha_{i2}^{G184S}$ mice on the HF diet also exhibit enhanced insulin sensitivity and increased glucose tolerance despite females having similar weight gain and adiposity compared to wild-type female mice.

Conclusions: RGS proteins and $G\alpha_{i2}$ signaling play a significant role in the control of insulin sensitivity and glucose metabolism. Identification of the specific RGS proteins involved might permit their consideration as potential therapeutic targets for obesity-related insulin resistance and type 2 diabetes.

INTRODUCTION

Obesity is reaching epidemic proportions world-wide (1). In the United States, obesity is the most common chronic disease, affecting more than 1 in 4 of all Americans, including children, and its incidence has been steadily increasing for the past 20 years (2). Obesity is a major risk factor for the development of insulin resistance and type 2 diabetes and is associated with hypertension and atherosclerosis (3). A better understanding of the biological, genetic and molecular mechanisms involved in obesity and diabetes is crucial for the development of new therapeutic strategies or antidiabetic drugs (4).

Obesity develops when energy intake exceeds energy expenditure. Regulation of food intake and energy expenditure is mainly controlled by neurons in the hypothalamus and brainstem with the help of several neuropeptides and neurotransmitters responding to long-term adipostatic signals (leptin and insulin) and to acute satiety/hunger hormones (ghrelin) (5). Many key receptors that mediate effects of neuropeptides and neurotransmitters involved in central regulation of energy balance are G protein-coupled receptors (GPCR) (6). Over 40 GPCRs have been implicated in regulation of body weight through mouse knockout and transgenic models, quantitative trait loci from crossbreeding experiments, and linkage studies (6; 7). The GPCRs involved in control of body weight and carbohydrate and lipid homeostasis are active targets in drug development. These include: the melanocortin system (8), NPY receptors (9), the cannabinoid system (10), monoamine GPCRs including serotonin, adrenergic and histamine receptors (11), the nicotinic acid receptor (12), and gut hormone systems including ghrelin and the glucagon-like peptide-1 receptor (13).

Most GPCRs signal through one or more G proteins, including those of $G\alpha_i$, $G\alpha_s$, and $G\alpha_q$ family members, to effectors such as adenylyl cyclases, phospholipase C, ion channels, and cGMP phosphodiesterase (14). Hormone-activated GPCRs promote guanine nucleotide exchange on the $G\alpha$ subunit then activated $G\alpha^{GTP}$ and $G\beta\gamma$ subunits dissociate to regulate their downstream effectors and cellular responses. GTP hydrolysis by $G\alpha$ restores the inactive heterotrimeric $G\alpha^{GDP}\beta\gamma$. Regulators of G protein signaling (RGS) proteins are a large and diverse family initially identified as GTPase activating proteins (GAPs) of heterotrimeric G protein $G\alpha$ subunits. They serve mostly negative modulatory roles in G protein-mediated signal transduction (15). Mechanistically, RGSs regulate GPCR responses by binding to and stimulating the GTPase activity of the receptor-activated GTP-bound $G\alpha$ subunits to rapidly deactivate $G\alpha$ (16). RGS proteins also can regulate G protein-effector interactions in other ways, either by competitively inhibiting $G\alpha$ binding to effectors such as phospholipase C or by serving as a protein adaptor which can recruit diverse effectors or regulators to the activated G proteins (17-19). To date, 37 human genes that encode proteins containing an RGS or RGS-like domain have been identified and most currently known RGS proteins regulate G_i and G_q signaling (20). The activity, sub-cellular distribution and expression levels of RGS proteins are dynamically regulated throughout development and in response to pharmacological or pathological alterations in cell signaling (21). Furthermore, there is emerging interest in RGS proteins as drug targets (18; 22; 23). However, the physiological functions of RGS proteins remain poorly defined. Little information is available implicating RGS proteins in control of metabolic activity to maintain body weight and energy balance (24; 25).

There are over 15 RGS proteins that can act on Gi/o family members (18). To overcome this redundancy of RGS function and to reveal the total contribution of RGS proteins in the regulation of any given G protein, we have utilized genomic knock-in animals expressing an RGS-insensitive allele, G184S, of $G\alpha_{i2}$ (26). The only known effect of the G184S mutation in $G\alpha$ is to disrupt RGS binding thus preventing GAP activity (27; 28). The knock-in approach maintains the normal distribution and level of $G\alpha_{i2}$ expression (26). As described previously, homozygous $G\alpha_{i2}^{G184S}$ mice display a complex phenotype that reveals a substantial role for RGS proteins acting on $G\alpha_{i2}$ (26; 29). They have enhanced signaling through $G\alpha_{i2}$, alterations in cardiac chronotropy and myelomonocytic cell numbers, and short stature. Based on preliminary observations that the mice were lean, we evaluate, in the present study, the influence of endogenous RGS protein action on $G\alpha_{i2}$ on fat deposits and glucose metabolism by placing RGS-insensitive $G\alpha_{i2}$ mice ($G\alpha_{i2}^{G184S}$ homozygotes) on high-fat (HF) or low-fat (LF) diets. Male $G\alpha_{i2}^{G184S}$ mice are resistant to HF-induced weight gain, demonstrate significantly decreased body fat, and are protected from insulin resistance. This appears to be a result of increased energy expenditure and is accompanied by enhanced peripheral insulin sensitivity. Interestingly, female $G\alpha_{i2}^{G184S}$ mice also exhibit enhanced insulin sensitivity and increased glucose tolerance despite weight gain and adiposity that is similar to wild-type females on the HF diet. These findings demonstrate that RGS proteins and $G\alpha_{i2}$ signaling play a critical role in the regulation of insulin sensitivity and glucose homeostasis *in vivo*. Furthermore, RGS proteins might provide new therapeutic opportunities for pharmacological prevention of HF diet-induced obesity and type 2 diabetes.

RESEARCH DESIGN AND METHODS

Animals. All protocols and procedures were approved by the University Committee on Use and Care of Animals and animal care was overseen by the Unit for Laboratory Animal Medicine (University of Michigan). The homozygous $G\alpha_{i2}^{G184S/G184S}$ mice (hereafter called $G\alpha_{i2}^{G184S}$) were previously described (26). Mice were originally obtained on a hybrid 129SVJ/C57Bl/6 background and were subsequently back-crossed at least 5 generations onto the C57BL/6J strain. Age- and gender-matched littermates were used for all experiments.

Study design. Female and male mice (wild-type and homozygous $G\alpha_{i2}^{G184S}$ mice) were maintained on a 12-h light/12-h dark schedule and fed standard laboratory chow and water *ad libitum*. Mutant mice shared the same cage with their littermate control. At 4 weeks of age, mice were randomly allocated to either a low-fat diet (10% fat, 70% carbohydrate, 20% protein; D12450B; Research Diets, New Brunswick, NJ) or a HF diet (45% fat, 35% carbohydrate, 20% protein, 4.7 kcal/g; D12451; Research Diets) for a period of 25 weeks.

Body weight was measured every other week. Food intake was measured at 10-11 weeks - each mouse was housed in an individual cage and food was weighed daily for 7 consecutive days. Energy expenditure was measured at 11 weeks and body composition (dual energy x-ray absorptiometry) was measured at 16 weeks. Glucose tolerance tests were performed at 16 and 25 weeks. Mice were sacrificed at 26 weeks after 5 h of food deprivation, serum was frozen, and tissues including four different fat depots were removed, weighed, and immediately frozen in liquid nitrogen for storage at -70°C or were fixed in 10% neutral buffered formalin for histological analysis.

Blood analysis. Glucose levels were determined in whole blood from mouse tails

using an Ascensis ELITE XL blood glucose meter (Bayer Corporation, Mishawaka, IN). Serum insulin and leptin were determined by enzyme-linked immunosorbent assay (CrystalChem, Downers Grove, IL).

Energy expenditure. Oxygen consumption (V_{O_2}) and carbon dioxide production (V_{CO_2}) were simultaneously measured by indirect calorimetry using the OxymaxTM System (Columbus Instruments, Columbus, OH). The mice were housed in the separate chambers with free access to food and water for 3 days for acclimatization before starting the measurements. Oxygen consumption was measured at 20 min intervals for a total of 20 hours and was normalized to body weight. Data during the light cycle, and the dark cycle were calculated separately.

Body fat analysis. Animals were anesthetized with isoflurane (5% for induction and 1 – 2% for maintenance), placed in the prone position, and scanned using dual energy x-ray absorptiometry on a Mouse Densitometer (PIXImus, GE Medical Systems). Body composition was estimated with pDEXA[®] SABRETM software. Measurements included total mass, total fat mass, total lean mass, and body fat percentage.

Glucose tolerance tests. Mice were fasted overnight for 14 h with free access to water. Each mouse then received an intraperitoneal injection of glucose (2 mg/g body wt). Blood samples were taken from the tail vein before glucose injection and at 15, 30, 60, 120 minutes after the injection and whole blood glucose was determined.

Insulin tolerance test. To measure the whole body insulin sensitivity, insulin tolerance tests were applied to 18-20 weeks old female mice after 5 weeks of HF-diet challenge. Mice were fasted for 4 hours with free access to water in the morning and then injected intraperitoneally with regular insulin (0.5 unit/kg body weight; Sigma). Tail-blood samples were obtained before insulin

administration (time-zero sample), as well as at 15, 30, 60 and 120 minutes after the insulin injection and whole blood glucose was determined.

Liver triglyceride (TG) measurement. Total liver lipids were extracted according to a method modified from that of Folch et al (30). Briefly, snap frozen liver tissues (70 mg) were homogenized in 1.5 ml of chloroform:methanol (2:1 v/v) solution and incubated at room temperature with shaking for 4 hours, then 0.75 ml of 0.1 M NaCl was added into the liver tissue homogenate. The organic phase was collected, dried and resuspended in 0.2 ml of 3 M KOH followed by incubating at 70°C for 1 hour to hydrolyze the triglycerides. Then 0.6 ml of 1 M $MgCl_2$ was added. Glycerol was measured using the Free Glycerol Reagent (Sigma). The amount of triglycerides was calculated by conversion from the glycerol content.

Statistical Analyses. Comparisons of individual group means used a 2-tailed Student's *t* test. To compare multiple data sets a 2-way ANOVA with Bonferroni post-test was used. All statistical calculations were done using GraphPad Prism version 4 (GraphPad Software Inc., San Diego).

RESULTS

Adult $G\alpha_{i2}^{G184S}$ mice have lower body weight and display subtle metabolic alterations on low fat or normal chow diets.

The most apparent physiological phenotype of the male and female homozygous RGS-insensitive $G\alpha_{i2}^{G184S}$ mice was their small size with significantly reduced body weight (Table 1 and ref. (26)). Early phenotyping studies on a mixed 129xC57/Bl/6J background showed individual mutant mice with dramatically reduced fat stores. However, after backcrossing onto the C57/Bl/6J background, the metabolic phenotype was significantly blunted. Female 20-22 week old $G\alpha_{i2}^{G184S}$ mutant mice fed normal chow showed only a non-significant trend toward decreased body

fat composition, as estimated with dual energy x-ray absorptiometry (Table 1). Consistent with reduced fat stores they also show slightly decreased serum leptin levels. Similar non-significant changes were seen in males (see below). In addition, both male and female $G\alpha_{i2}^{G184S}$ mutants fed normal chow (or on a LF diet) also had a tendency toward decreased non-fasting serum insulin levels (Fig S1) but they had minimal differences in glucose tolerance compared to wild-type animals. Thus to bring out a latent phenotype we put the mice on a high-fat diet.

$G\alpha_{i2}^{G184S}$ males are protected from diet-induced obesity.

HF feeding has been demonstrated to induce body weight gain and obesity and is associated with insulin resistance. We analyzed the effect of HF or LF diet on $G\alpha_{i2}^{G184S}$ homozygous and littermate control mice for 25 weeks. Control mice on either diet had higher body weights compared to mutants during whole diet period (Fig. 1A in male and B in female) so initial body weight was subtracted to determine weight gain (Fig. 1C and D). As expected, WT male mice showed a rapid weight gain on HF compared to LF diet. Surprisingly, mutant male mice fed the HF-diet did not gain any more weight than did those on the LF-diet (Fig. 1C). The difference in weight gain between male WT and $G\alpha_{i2}^{G184S}$ mice on HF-diet was highly significant (Fig. 1C, weeks 22-26) but weight gains for male WT and $G\alpha_{i2}^{G184S}$ mice on the LF-diet were comparable. In contrast to the males, female $G\alpha_{i2}^{G184S}$ mice exhibited similar weight gains on both diets compared to WT females (Fig. 1D).

Reduced body fat mass and hepatic triglyceride in male $G\alpha_{i2}^{G184S}$ mice.

We asked whether the reduced weight gain in male mutant mice was associated with alterations in adiposity. At necropsy, male $G\alpha_{i2}^{G184S}$ mice showed substantial reductions in the size of fat pads compared to HF-fed WT mice (Fig. 2A). Body composition

analysis by dual energy x-ray absorptiometry (DEXA) showed an ~ 30% reduction in percent body fat by DEXA in mutant males compared to control mice after 16-weeks on the HF diet ($P < 0.05$, Fig. 2B), however, no significant difference was observed in lean mass (data not shown). Consistent with the reduced adipose stores, there is a dramatically decreased leptin level in HF-fed male $G\alpha_{i2}^{G184S}$ mice ($P < 0.001$, Fig. 2C). Females showed minor, non-significant changes in adiposity and a trend toward decreased leptin levels, but no significant effect of genotype on either parameter (Fig. S2). Furthermore at the end of the diet study, HF-fed mutant male mice showed markedly reduced white adipose tissue mass including inguinal and perirenal fat ($P < 0.05$, Table. 2). In contrast, brown adipose tissue (interscapular fat) and most other tissues (such as kidney) retained their normal proportion to body weight (Table. 2). Interestingly, male mutant mice fed the LF-diet also showed less epididymal fat than controls ($P < 0.05$, Table. 2), but no significant difference in other fat tissues.

In addition, male mutant mice have significantly lower liver weights and liver triglyceride levels than those of WT mice (Fig. 3C). Furthermore, liver histology in control mice (Fig. 3A and B) exhibited vacuoles indicating increased hepatic lipid accumulation after 25 weeks of HF-diet, while mutant mice had no appearance of vacuoles.

Increased metabolic rate relative to body weight in male $G\alpha_{i2}^{G184S}$ mice.

We further investigated whether the resistance to diet-induced obesity observed in male mutant mice resulted from decreased food intake or increased energy expenditure. Food intake was measured for a continuous seven day period for each group of mice after 10 weeks on LF or HF diet feeding. Food intake normalized to body weight was similar in the $G\alpha_{i2}^{G184S}$ and wild-type mice on the HF and LF diet (Fig. 4A and S3A). Indeed, energy

consumption per gram of body weight was slightly, but not significantly, higher in both male and female mutant mice so reduced caloric intake alone does not appear to explain the reduced weight gain in males on HF diet. After 11 weeks on HF diet feeding, male $G\alpha_{i2}^{G184S}$ mice exhibited significantly higher rates of total and night-time O_2 consumption per g body weight than did wild-type mice (Fig. 4B and C). While absolute O_2 consumption was reduced (to 83 % of wt) due to the smaller stature of the mutant mice, the absolute food intake (77% of wt) was reduced even more than was O_2 consumption leading to a relatively higher energy utilization vs intake. In addition to the greater night-time VO_2 , there was also a small, but not statistically significant, increase in VO_2 in the light period which could represent either changes in basal metabolic rate or in daytime activity. In contrast, O_2 consumption in the female mutant mice (normalized to body weight) was not different from wild-type either on LF or HF diet (Fig. S3B & C) consistent with their equivalent weight gain on HF diet. Males on LF diet also did not show any increase in VO_2 . (Fig. S4) All mice (male/female, LF/HF) showed a normal diurnal pattern with higher VO_2 during the night (active state) and lower rates during the day (resting state). The increased O_2 consumption in males may in part be related to our previously reported finding of increased activity in a telemetry study (26). Measurements of urine catecholamines revealed a modest increase in norepinephrine excretion in G184S male, but not female, mice on a normal chow diet (Fig. S5).

Improved whole body insulin sensitivity in $G\alpha_{i2}^{G184S}$ mice.

The effect of HF diet on insulin levels and glucose tolerance was assessed in $G\alpha_{i2}^{G184S}$ and littermate control mice. Blood glucose and insulin levels were measured after 5 hours of fasting. On the LF-diet, $G\alpha_{i2}^{G184S}$ mice (male and female) showed slightly, but not

significantly, reduced fasting plasma insulin levels compared to the WT mice (Fig. S1 and see also Table 1). After 25 weeks of HF feeding, both male and female $G\alpha_{i2}^{G184S}$ mice showed markedly lower fasting insulin levels than did control mice (Fig. 5B). Fasting blood glucose concentrations did not differ significantly despite the lower insulin levels in the mutants (Fig. 5A). To investigate whether the changes in plasma insulin levels upon HF diet feeding were associated with improved glucose handling, glucose tolerance tests were performed by i.p. administration of 2 mg glucose/g body weight. As shown in Fig. 5 both male and female $G\alpha_{i2}^{G184S}$ mice on the HF diet were more efficient in clearing a bolus of glucose than the control mice. Two way ANOVA showed a highly significant genotype effect with significant decreases in glucose at 60 and 120 minutes for males and 60 minutes for females. The area under the curve of glucose levels were reduced 28% in males and 22% in females (Fig. S6). Taken together, the low insulin, normal glucose, and improved glucose tolerance displayed by the $G\alpha_{i2}^{G184S}$ mice maintained on the HF-diet indicate significantly enhanced insulin sensitivity. While an enhanced insulin sensitivity is expected in $G\alpha_{i2}^{G184S}$ males, where the weight gain and adiposity was significantly reduced, it was unexpected in females. Furthermore, this enhanced insulin sensitivity in female $G\alpha_{i2}^{G184S}$ mice was confirmed in insulin tolerance tests after only a 5 week HF-diet challenge (Fig. 5E). Blood glucose levels in female mutant mice fell by 50% over a one hour period following an intraperitoneal injection of insulin (0.5 unit/kg body weight) but dropped only by about 25% in WT mice (Fig. 5E and see Fig S6C). The reciprocal of the AUC was 21% greater in the $G\alpha_{i2}^{G184S}$ mutants compared to WT females consistent with an increase in insulin sensitivity.

DISCUSSION

RGS proteins speed the turn-off of G protein signals and inhibit G_i and G_q -mediated signal transduction but the *in vivo* roles of RGS proteins remain poorly defined. Given the important role of G protein signaling, the goal of this study was to analyze the metabolic alterations of RGS-insensitive $G\alpha_{i2}^{G184S}$ mice to reveal the total contribution of RGS regulation at the $G\alpha_{i2}$ subunit in the regulation of body weight and glucose homeostasis. Male $G\alpha_{i2}^{G184S}$ homozygous mice are resistant to HF-induced weight gain and have significantly decreased body fat due to increased energy expenditure and also show enhanced insulin sensitivity. Interestingly, female $G\alpha_{i2}^{G184S}$ homozygous mice also exhibit increased glucose tolerance and enhanced insulin sensitivity despite having a similar weight gain and adiposity compared to wild-type mice on the HF diet. These alterations were only revealed after an adipogenic dietary challenge because the RGS-insensitive mutation in $G\alpha_{i2}$ had minimal effects on fat mass or glucose metabolism in adult mice fed a normal chow or low-fat diet. The RGS-insensitive $G\alpha_{i2}^{G184S}$ mice should have enhanced, but receptor-dependent, G_{i2} activity in any tissue with functional RGS activity, thus the adipogenic diet may activate signals that stimulate some important $G\alpha_{i2}$ -coupled receptors to prevent the weight gain and improve glucose tolerance.

$G\alpha_{i2}$ expression is ubiquitous in peripheral tissues and is also present in many brain regions including hypothalamus. At this point, we do not know if the primary locus of effects seen in this model are central or peripheral but G proteins clearly play important central roles in both general behavioral functions and in central control of metabolic processes mediated by a large number of G protein coupled receptors (6). Fat deposits are significantly reduced in male $G\alpha_{i2}^{G184S}$ mice on the HF-diet and this is

associated with elevated energy expenditure. Since $G\alpha_{i2}^{G184S}$ mice on a low fat diet do not show significantly increased VO_2 it appears that the adipogenic diet somehow leads to enhanced signaling through one or more G_i -coupled receptor. This could be due to increased physical activity or central control of energy utilization. It is well known that lean animals generally exhibit an increase in insulin sensitivity so it is not surprising that the male mice show enhanced insulin sensitivity. However, female $G\alpha_{i2}^{G184S}$ mice also exhibit enhanced insulin sensitivity despite having similar weight gain and adiposity to those of wild-type female mice on the HF diet. While it is not clear why we see sex differences, it is commonly observed that males and females respond differently to high fat diets and a recent study also found sex differences in regulation of the NPY signaling system in the hypothalamus (31). Regardless, it is likely that the increased insulin sensitivity displayed by the $G\alpha_{i2}^{G184S}$ mice includes a significant role of $G\alpha_{i2}$ -mediated signaling in regulating glucose utilization or other metabolic functions and is not simply due to a reduction in body fat mass. This observation may explain why the decreased insulin levels in $G\alpha_{i2}^{G184S}$ mice do not result in hyperglycemia and/or impaired glucose tolerance.

$G\alpha_{i2}$ also plays a significant role in adipose tissue function. Many $G\alpha_i$ -coupled receptors have strong anti-lipolytic actions mediated by pertussis-toxin (PTX) sensitive mechanisms (32), including α_{2a} -adrenergic, A1 adenosine, and prostaglandin receptors, and the recently deorphanized receptor for nicotinic acid (12). However, this might be expected to lead to enhanced accumulation of fat in our mice (33). In addition to its antilipolytic actions, $G\alpha_i$ signaling has been demonstrated to play a role in fat cell differentiation. $G\alpha_{i2}$ mRNA levels decrease during 3T3-L1 preadipocyte differentiation into adipocytes (34) and nearly all cocktails

used to induce adipocyte differentiation in culture include the adenosine antagonist, IBMX, suggesting that G_i activation (perhaps by reducing cAMP) prevents differentiation. Activation of G_i -coupled LPA1 receptors or over-expression of A1 adenosine receptors reduced differentiation of preadipocytes (35; 36); and LPA1 receptor deficient mice become obese (35). Thus, reduced adipose differentiation could play a role in the reduced fat phenotype.

Besides direct actions on fat tissue, there are other potential peripheral mechanisms for the decreased fat mass in the $G\alpha_{i2}^{G184S}$ mice. Hepatic G_s -signaling pathways play a role in glucose and lipid regulation (37). Liver-specific $G\alpha_s$ knockout mice had increased liver weight and glycogen content but had reduced adiposity and increased glucose uptake in liver and muscle. Thus enhanced $G\alpha_{i2}$ signaling in liver with reduced cAMP levels could produce a similar phenotype however effects of $G\alpha_s$ disruption systemically are quite complex due to imprinting at the *GNAS* locus with either hypermetabolic or obesity phenotypes depending on maternal or paternal inheritance (38). In addition, increased sympathetic tone could lead to reduced adiposity. Male $G\alpha_{i2}^{G184S}$ mice exhibited increased heart rate and physical activity during telemetry ECG monitoring (26), consistent with a contribution of central sympathetic activation. Indeed, we see (Fig. S5) an increase in urinary norepinephrine excretion in male, but not female, $G184S$ mice which correlates with their lower adiposity.

What could account for enhanced insulin responses in these mice? In the periphery, glucose uptake into skeletal muscle and adipocytes is mediated by translocation of the glucose transporter subtype 4 (GLUT4) from intracellular vesicles to the plasma membrane (39). A variety of GPCRs have been demonstrated to modify insulin-induced GLUT4 translocation. G_s -mediated signaling

negatively regulates insulin-induced GLUT4 translocation. Heterozygous *GNAS*-deficient mice show an increased insulin sensitivity due to enhanced insulin-dependent glucose uptake into the skeletal muscle (40; 41). Consistent with this, transgenic mice expressing a constitutively active mutant of $G\alpha_s$ Q227L in fat, liver, and skeletal muscle showed decreased glucose tolerance (42). In contrast, G_i family G proteins seem to facilitate insulin actions. Pretreatment of isolated adipocytes and soleus muscle with PTX results in reduced insulin-stimulated glucose uptake (43). When $G\alpha_{i2}$ was down-regulated in liver and adipose tissue using an antisense RNA approach, insulin resistance developed due to increased protein tyrosine phosphatase-1B (PTP-1B) activity (44). Additionally, mice expressing a constitutively active mutant of $G\alpha_{i2}$ Q205L in fat, liver, and skeletal muscle displayed reduced fasting blood glucose levels and increased glucose tolerance (45). Adipocytes from these mice showed enhanced insulin-induced glucose uptake and GLUT4 translocation, as well as increased PI3-kinase and Akt activities (46). Interestingly, we previously showed that embryo fibroblasts from our mice had enhanced Akt activation by lysophosphatidic acid (30). Furthermore, ligand-dependent autophosphorylation of the human insulin receptor is positively regulated by $G\alpha_{i2}$ proteins (47). However, it is still not clear at which level the insulin receptor-mediated pathway interacts with the G_i -mediated pathway. It has even been suggested that G_i/G_o family G proteins physically interact with insulin receptor (48). Therefore, our results are consistent with this rather large – but not often appreciated body of literature indicating important contributions of $G\alpha_{i2}$ in metabolic regulation. The RGS-insensitive $G\alpha_{i2}^{G184S}$ mice described here provide a more physiological model to dissect the mechanism of interaction between $G\alpha_{i2}$ protein signaling and insulin actions *in vivo*. Identifying the tissue site of $G\alpha_{i2}^{G184S}$ effects, such as in

adipocytes, muscle, or brain, where hypothalamic insulin action via the PI3K may contribute to glucose homeostasis (49), will be an important future goal.

In conclusion, we have demonstrated that RGS-insensitive $G\alpha_{i2}^{G184S}$ mice exhibit resistance to the metabolic effects of a HF-diet. Male $G\alpha_{i2}^{G184S}$ mice show a reduction in body weight and body fat mass, while males as well as females show improved glucose tolerance and insulin sensitivity. Future studies will aim to explore the molecular basis underlying this phenomenon. As a first step toward understanding the role of the RGS proteins and $G\alpha_{i2}$ signaling in body weight and glucose homeostasis, it will be important to identify the specific tissues and RGS proteins mediating these effects. This could open the door to a role for pharmacologic inhibition of RGS proteins as one element in control of obesity and impaired glucose

tolerance associated with the chronic intake of fatty diets.

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TABLE 1Metabolic phenotyping of female homozygous $G\alpha_{i2}^{G184S/G184S}$ mice on the normal mice chow

	Wild-type	$G\alpha_{i2}^{GS/GS}$
<i>n</i>	9	10
Body Weight (g)	24.5 ± 0.7	19.7 ± 0.5***
Serum Glucose (mg/dl)		
Overnight fasting	96 ± 5	107 ± 11
Non-fasting	153 ± 15	136 ± 8
Insulin (ng/ml)	0.23 ± 0.03	0.17 ± 0.02
Fat mass (%)	17.1 ± 0.8	15.3 ± 0.8
Plasma leptin (ng/ml)	4.3 ± 0.6	2.9 ± 0.6

Data are mean ± SE. Female mice were placed on normal chow for 20-22 weeks, after which weight, glucose (fasting or non-fasting), insulin (non-fasting) and leptin were measured. Fat mass was estimated with dual energy x-ray absorptiometry. ***P < 0.0001 by t test.

TABLE 2

Weights of adipose tissue and other organs normalized to body weight in male $G\alpha_{i2}^{G184S/G184S}$ and control mice after 25 week diet challenge

Mice	Male LF		Male HF	
	Wild-type	GS/GS	Wild-type	GS/GS
White adipose tissue				
Epididymal	42.4 ± 5.4	28.8 ± 7.8 *	43.7 ± 6.4	33.2 ± 7.3
Inguinal	25.7 ± 3.6	21.7 ± 6.8	43.4 ± 2.2	28.1 ± 5.9 *
Perirenal	19.5 ± 2.7	11.8 ± 3.9	32.4 ± 1.3	17.5 ± 2.9 *
Brown adipose tissue	13 ± 1.4	10.4 ± 2.1	15.3 ± 0.7	11.1 ± 1.4
Pancreas	7.9 ± 0.5	7.4 ± 0.4	7.2 ± 0.5	8.8 ± 1.2
Kidney	10.8 ± 0.6	11.7 ± 0.5	10.4 ± 0.7	11.7 ± 1.1
Lung	4.6 ± 0.3	5.3 ± 0.5	3.6 ± 0.1	4.9 ± 0.2

Normalized fat pads and organ weights as percentages of body weight (mg/g). Male wild-type (WT) and homozygous (GS/GS) mice were sacrificed after 25 week diet challenges. Significant differences compared with wild-type mice: * $P < 0.05$ by 2-way ANOVA with Bonferroni post-test. Data are means ± SE; $n = 5$ mice/group.

Figure legends

FIG. 1. Decreased body weight in $G\alpha_{i2}^{G184S}$ homozygous males during the feeding study. *A* and *B*: Body weight in male and female. $G\alpha_{i2}^{G184S}$ homozygous (GS/GS) mice have significantly lower body weight than wild type (WT) during both high-fat (HF) or low-fat (LF) feeding periods. Genotype effect: $P < 0.0001$ by 2-way ANOVA. *C* and *D*: Weight gains in male and female. Comparing WT and GS/GS on HF-diet feeding: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by 2-way ANOVA with Bonferroni post-test. Comparing WT and GS/GS on LF-diet feeding: # $P < 0.05$, ## $P < 0.01$ by 2-way ANOVA with Bonferroni post-test. Data are means \pm SE; $n = 5$ mice/group.

FIG. 2. Decreased adiposity in male $G\alpha_{i2}^{G184S}$ homozygote (GS/GS). *A*: Exposed ventral view of male mice on 25 weeks HF-diet (arrows: epididymal fat pads, arrow heads: dorsolumbar fat pads). *B*. Fat mass calculated by dual-energy x-ray absorptiometry after 16 weeks on high-fat (HF) or low-fat (LF) diet. *C*: 5- hour fasting leptin concentration after a 25 week diet challenge. # $P < 0.05$, ## $P < 0.01$ different from LF by *t* test; * $P < 0.05$, *** $P < 0.001$ different from WT by *t* test. Data are means \pm SE; $n = 5$ mice/group.

FIG. 3. Reduced hepatic steatosis in male $G\alpha_{i2}^{G184S}$ homozygotes (GS/GS). *A* and *B*: Histological analysis of the livers in male $G\alpha_{i2}^{G184S}$ homozygote and control mice. Representative H&E stained liver sections from wild-type (WT, *A*) and $G\alpha_{i2}^{G184S}$ homozygote (GS/GS, *B*) after 25 weeks high-fat diet challenge. *C*: Liver weight and hepatic triglycerides. * $P < 0.05$ by *t* test. Data are means and the actual scatter; $n = 4- 5$ mice/group.

FIG. 4. Energy homeostasis of male wild-type (WT) and $G\alpha_{i2}^{G184S}$ homozygote (GS/GS) fed high-fat (HF) or low-fat (LF) diets. *A*: Daily food intake measured over 7 days. *B* and *C*: Time course and summary of total oxygen consumption rate (VO_2) measured during the dark and light periods. Genotype effect: $P < 0.0001$ by 2-way ANOVA. * $P < 0.05$, ** $P < 0.01$ by 2-way ANOVA with Bonferroni post-test, significant differences at specific times are marked. # $P < 0.05$ by *t* test. Data are means \pm SE; $n = 5$ mice/group.

FIG. 5. Improved insulin sensitivity in $G\alpha_{i2}^{G184S}$ homozygote (GS/GS) mice. After a 25 week HF diet challenge, blood glucose (*A*) or plasma insulin concentration (*B*) in mice were measured as described in Methods after 5-hours fasting. Intraperitoneal glucose tolerance test in male (*C*) and female (*D*) mice was also measured. Mice were fasted overnight, then glucose was injected intraperitoneally (2 mg/g body weight). (*E*) Insulin tolerance tests were performed on 20 weeks old female mice after a 5 week HF-diet challenge. Mice were fasted for 4 hours in the morning and injected intraperitoneally with regular insulin (0.5 unit/kg body weight). Changes in blood glucose were monitored over time. Genotype effect: ** $P < 0.001$, *** $P < 0.0001$ by 2-way ANOVA. Significant differences at specific time are marked, * $P < 0.05$, ** $P < 0.01$, by 2-way ANOVA with Bonferroni post-test, # $P < 0.05$, ## $P < 0.01$ by *t* test. Data are means \pm SE; $n = 3-7$ mice/group.

Figure 1

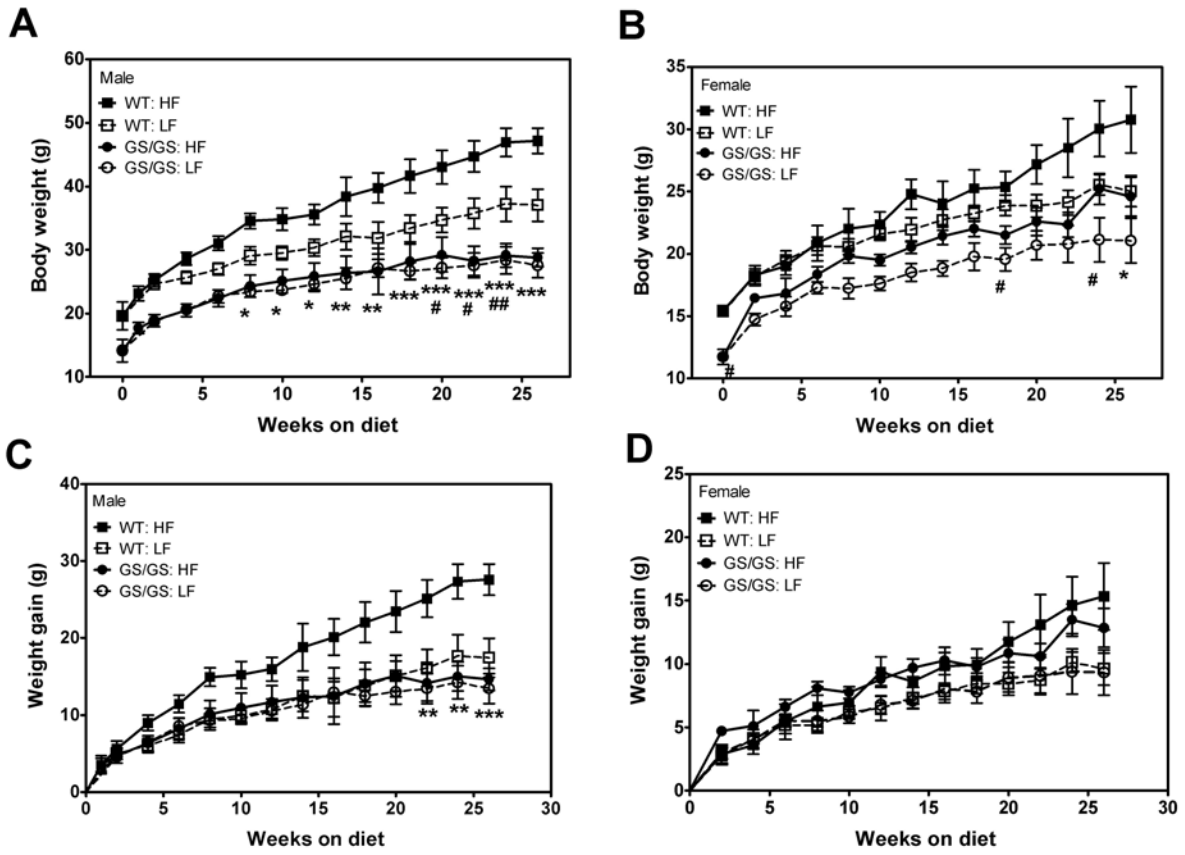


Figure 2

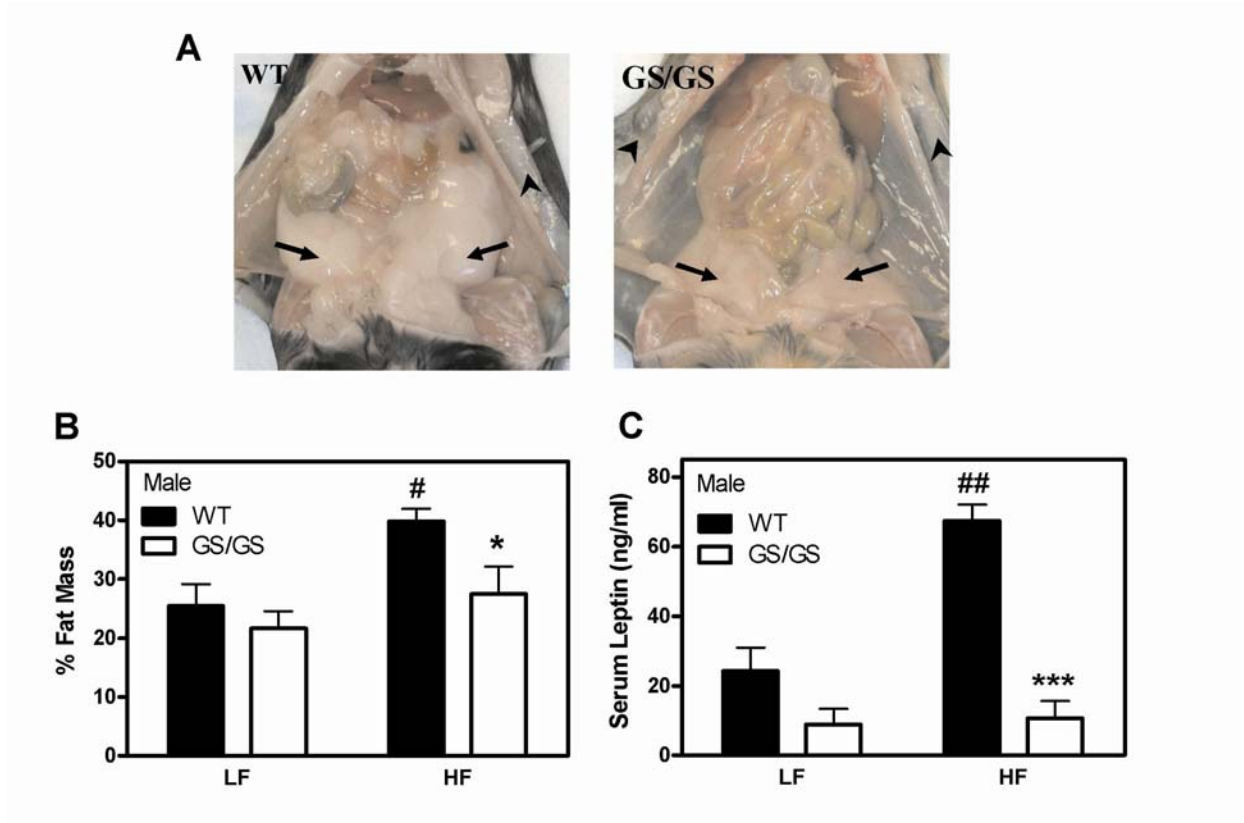


Figure 3

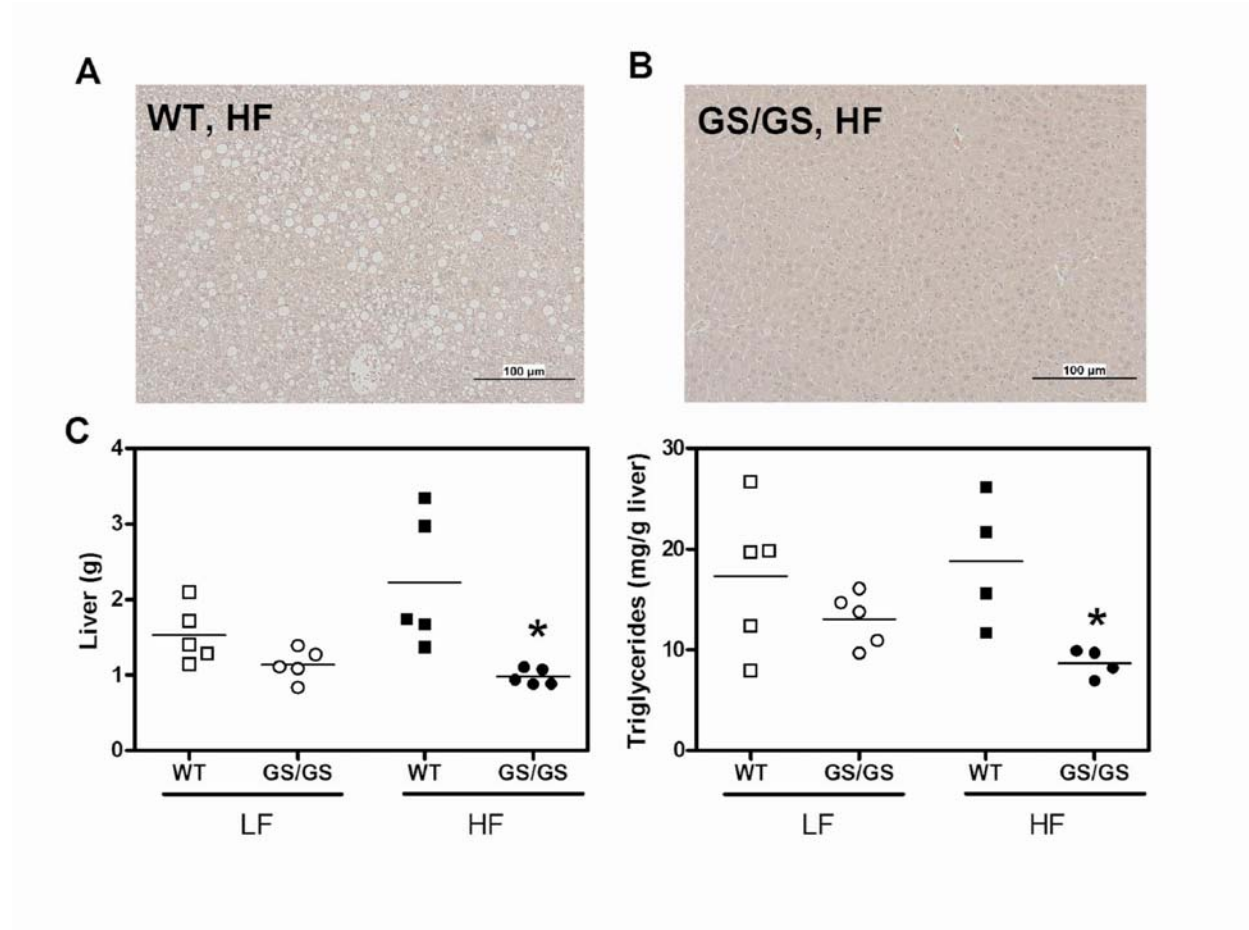


Figure 4

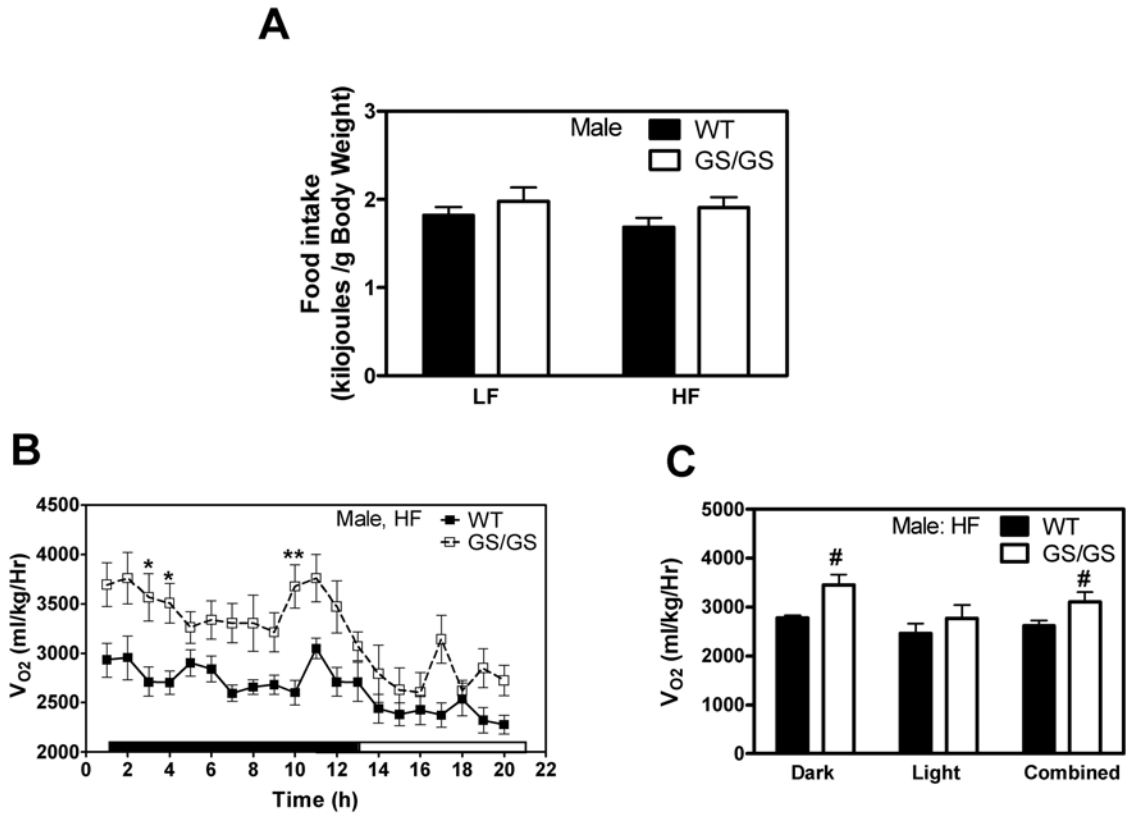


Figure 5

