Cellular Signalling 23 (2011) 1375–1386

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

Resistance to age-related, normal body weight gain in RGS2 deficient mice

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ARTICLE INFO

Article history:
Received 13 November 2010
Received in revised form 1 March 2011
Accepted 21 March 2011
Available online 5 April 2011

Keywords:
RGS protein
RGS2
G protein
Adipocyte differentiation
Lean mouse model

ABSTRACT

RGS2 (regulator of G protein signaling 2) is known to limit signals mediated via Gq- and Gs-coupled GPCRs (G protein coupled receptors), and it has been implicated in the differentiation of several cells types. The physiology of RGS2 knockout mice ($rgs2^{-/-}$) has been studied in some detail, however, a metabolic phenotype has not previously been reported. We observed that old (21–24 month) $rgs2^{-/-}$ mice weigh much less than wild-type C57BL/6 controls, and exhibit greatly reduced fat deposits, decreased serum lipids, and low leptin levels. Lower weight was evident as early as four weeks and continued throughout life. Younger adult male $rgs2^{-/-}$ mice (4–8 months) were found to show similar strain-related differences as the aged animals, as well improved glucose clearance and insulin sensitivity, and enhanced beta-adrenergic and glucagon signaling in isolated hepatocytes. In addition, $rgs2^{-/-}$ pre-adipocytes had reduced levels of differentiation markers (Peroxisome proliferator-activated receptor γ (PPARγ); lipoprotein lipase (Lpl); CCAAT/enhancer binding protein α (CEBPα)) and also $rgs2^{-/-}$ white adipocytes were small relative to controls, suggesting altered adipogenesis. In wild-type animals, RGS2 mRNA was decreased in brown adipose tissue after cold exposure (7 h at 4 °C) but increased in white adipose tissue in response to a high fat diet, also suggesting a role in lipid storage. No differences between strains were detected with respect to food intake, energy expenditure, GPCR-stimulated lipolysis, or adaptive thermogenesis. In conclusion this study points to RGS2 as being an important regulatory factor in controlling body weight and adipose function.

1. Introduction

RGS (regulator of G protein signaling) proteins are GTPase activating proteins (GAPs) which accelerate the termination of signaling via G protein coupled receptors (GPCRs), limiting the intensity and duration of their signals. All RGS proteins contain an approximately 120 amino acid RGS domain which binds to activated (GTP-bound) heterotrimeric G proteins, accelerating their intrinsic GTPase activity (hydrolysis of bound GTP to GDP), and reverting the G protein to its inactive (GDP-bound) state. Some RGS proteins can also limit GPCR signaling via GAP-independent mechanisms such as effector antagonism and guanine nucleotide dissociation inhibition [1].

RGS2 is a member of the B/R4 family of RGS proteins which have short, simple amino and carboxy terminal domains. RGS2 is unique among RGS proteins due to its selectivity for Gαq over Gαi/o subunits [2,3], and its ability to inhibit signals through Gs-coupled receptors via an apparently GAP-independent mechanism [4]. RGS2 is ubiquitously expressed at low levels under basal conditions [5] and is considered to be a primary response gene whose expression is transiently and rapidly induced following changes in homeostasis through GPCR-dependent [6–10] and -independent mechanisms [11,12].

Through its GAP and non-GAP effects RGS2 has been shown to be able to inhibit signaling through Gq- [2,8,13], Gs- [4,9,10,14] and Gi/o- [2,6] coupled receptors, although it is not clear to what extent interactions with the latter G protein subfamily occur in vivo. In vitro studies have demonstrated that inhibition of GPCR signaling by RGS2 is important in mediating GIP (glucose-dependent insulinotropic polypeptide)-induced insulin release from pancreatic β-cells [14], cardiomyocyte hypertrophy [8,15], GLUT-4 insulin-responsive glucose transporter translocation in adipocytes [16] and short-term synaptic plasticity in hippocampal neurons [17]. In addition RGS2 is able to modulate other processes including transient receptor potential vanilloid type 6 (TRPV6) Ca^{2+} channel activity [18], microtubule polymerization [19], and mRNA translation into protein [20]. RGS2 has also been implicated, albeit through unknown mechanisms, in the differentiation of a variety of cell types [21–23] and indeed it is thought to play an important role in the maturation of adipocytes. RGS2 is rapidly and transiently upregulated in differentiating mouse 3T3-L1 preadipocyte cells [24], while ectopic

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doi:10.1016/j.cellsig.2011.03.020

expression of RGS2 in mouse NIH-3T3 preadipocyte cells promotes differentiation to adipocytes and expression of adipogenic markers [23]. Furthermore one recent study found that RGS2 is rapidly and persistently (up to 9 days) induced in differentiating 3T3-L1 pre-adipocytes in a cAMP-dependent manner and that this was due to activation of an Sp-1, but not C/EBP (CCAAT/enhancer binding protein) response element within the RGS2 promoter sequence [25].

An *rgs2*$^{-/-}$ mouse model has been generated to examine the physiological actions of RGS2. These mice develop normally at the expected Mendelian frequencies and are apparently healthy and fertile with normal circadian activity and motor function [26]. Observed phenotypes include decreased male aggression, increased anxiety, reduced T cell proliferation [26], and increased renal responsiveness to vasopressin [27]. Furthermore these mice exhibit a mild to moderate hypertensive phenotype which is attributed to prolonged vasoconstrictor signaling, decreased cGMP-mediated vascular relaxation and elevated sympathetic nervous system activity [28–30], and they also have increased sensitivity to pressure-induced cardiac hypertrophy [31].

Studies on *rgs2*$^{-/-}$ mice have so far been carried out on healthy young animals. We hypothesized that a functional phenotype of the *rgs2*$^{-/-}$ mouse might manifest itself as the animals aged. Thus we kept a group of *rgs2*$^{-/-}$ and wild-type animals to the age of two years. We report in this study that as these mice age they develop a very significant weight-related phenotype wherein they fail to exhibit the normal body fat gain of sedentary, free feeding laboratory animals. Upon further examination it is apparent that younger mice also weigh less than wild-type and this is accompanied by reduced serum lipids, increased insulin sensitivity and smaller adipocytes. Unexpectedly however, the mice are not resistant to diet-induced obesity.

2. Materials and methods

2.1. *rgs2*$^{-/-}$ mice

The generation and genotyping of *rgs2*$^{-/-}$ mice have been described [9,26]. Briefly, animals were from congenic *rgs2*$^{-/-}$ and wild-type control (*rgs2*$^{+/+}$) C57BL/6 colonies previously established in our laboratory, as described [9]. Mice were provided ad libitum with water and food and subjected to a standard (12 h:12 h) light/dark cycle. Animals were maintained in accordance with the Institute of Laboratory Animal Research Guide for the Care and Use of Laboratory Animals. The studies were approved by the Council on Animal Care at the University of Western Ontario and complied with the guidelines of the Canadian Council on Animal Care. For high fat diet studies, 9-week-old male mice were housed in pairs and fed either standard mouse chow (2018; Harlan Teklad, 17% calories from fat, 23% calories from protein and 60% calories from carbohydrate) or high fat diet (TD 88137; Harlan Teklad, 42% calories from fat, 15.3% calories from protein and 42.7% calories from carbohydrate). Body and food weight were determined once per week. After 25 weeks on the diet lean and fat mass of mice were determined using a magnetic resonance body composition analyzer (Echo Medical Systems, LLC, Houston, TX) before sacrifice and blood and tissue collection.

2.2. Quantification of serum lipids and bioanalytes

Blood samples were allowed to clot for 30 min before being centrifuged (2700×*g*, 10 min) and serum removed and stored at −80 °C. Free fatty acids, cholesterol and triglycerides were assayed by colorimetric kits (Wako Diagnostics, Richmond, VA) using a microplate spectrophotometer. Levels of bioanalytes were elucidated using multiplexed biomarker immunoassay kits (Linco*plex*, Millipore Corp, MA). A Bio-Plex™ 200 readout System was used (Bio-Rad Laboratories, Hercules, CA), which utilizes Luminex® xMAP™ fluorescent bead-based technology (Luminex Corp., Austin, TX). Levels were automatically calculated from standard curves using Bio-Plex Manager software (v.4.1.1, Bio-Rad).

2.3. Histological examinations

Paraffin-embedded sections of tissues were prepared and stained with hematoyxlin and eosin. White adipose cell size was quantified by determining the average cell area at 20× magnification using a Northern Eclipse (Empix Imaging Mississauga, ON) image analysis system. For each animal 4 random fields were measured and averaged.

2.4. Pre-adipocyte isolation and in vitro adipoctye differentiation

Pre-adipocytes were isolated from three-month-old male mice: Epididymal and retroperitoneal fatpads were collected, dissected from blood vessels, weighed, finely minced and treated with digestion buffer (3 ml/g tissue) (0.5 mg/ml collagenase class IV and 1.5% bovine serum albumin (BSA) (Sigma, Oakville, ON, Canada) for 40 min at 37 °C. The digested material was filtered through 100 μm nylon mesh to remove debris and centrifuged at 600×*g* for 5 min. The cell pellet was washed by resuspending and centrifuging three times with PBS containing 10% FBS, filtered through 20 μm nylon mesh and then centrifuged. The pelleted pre-adipocytes were resuspended in culture medium (DMED/F12 (Invitrogen Life Technologies), and 10% FBS). Pre-adipocytes were seeded in 24 well plates and cultured in a humidified incubator at 37 °C, in the presence of 5% CO_2.

Two days after isolated pre-adipocytes reached confluence (day 0), they were stimulated to differentiate by the addition of a cocktail containing 1 μM dexamethasone (Dex) (Sigma, Oakville, ON, Canada), 0.5 mM IBMX and 1 μg/ml insulin (Sigma, Oakville, ON, Canada). After 48 h, medium was replaced with culture medium plus insulin only, after which cells were given fresh medium every 2 days. Differentiated primary pre-adipocytes and vehicle-treated control cells were harvested at day 8. Expression of differentiation marker genes (peroxisome proliferator-activated receptor γ (PPAR γ), Sterol Regulatory Element Binding Protein (SREBP), lipoprotein lipase (LPL) and CCAAT-enhancer-binding protein α (CEBP α)) was examined using real-time PCR.

2.5. Hepatocyte isolation and intracellular cAMP determination

Hepatocytes were isolated from young adult (1–3 month old) male mice: Mice were anesthetized with ketamine/xylazine (100 mg/kg, 5 mg/kg respectively), and livers were exposed and perfused through the vena cava with Ca^{2+}-free Hanks' balanced solution at 5 ml/min for 8 min, followed by serum-free Williams' medium containing collagenase (type II, 50 U/ml, Worthington Biochemical Corp, Lakewood, NJ, USA), HEPES (10 mM), and NaOH (0.004 N) at 5 ml/min for 12 min. Livers were then removed and hepatocytes separated by Percoll gradient separation. Isolated cells were cultured in Williams' medium with 10% FBS for 24 h before treatment with isoproterenol or glucagon (plus the phosphodiesterase inhibitor IBMX) for 90 s and lysis. cAMP levels were determined using the cAMP Biotrak enzyme immunoassay system (GE healthcare, Baie d'Urfé, Québec, Canada).

2.6. Glucose production measurement and phospho-glycogen synthase level determination

Isolated hepatocytes were prepared as above from WT and *rgs2*$^{-/-}$ mice. The culture medium was replaced with fresh Williams' medium containing 10 nM dexamethasone and 10 nM insulin 3 h after isolation. After 16 h incubation, cells were washed 3 times with PBS to remove extra glucose before treatment with 10 μM isoproterenol or 100 nM glucagon in the presence of IBMX using glucose-free, phenol red-free DMEM medium ((Sigma, Oakville, ON, Canada)) containing gluconeogensis substrates (2 mM sodium lactate and 20 mM sodium pyruvate). Glucose levels in the culture medium were measured 1 h

after treatment using a glucose assay kit from Roche Applied Science (cat. No. 0716251) and normalized to protein concentrations. Cell lysates were separated by SDS-PAGE, transferred to PVDF membranes and used to estimate phosphorylated (Ser641) and total glycogen synthase levels by Western blotting, as described below.

2.7. Calorimetry

Measurements of metabolic rate were made using open flow respirometry (Sable system FC-1B O_2 analyzer). The set up consisted of 4 chambers maintained at thermoneutrality (28 °C), each housing one mouse, plus one reference channel for baseline measurements. O_2 readings were taken every second for 10 min per channel with 2 min pauses between channels. Mice were allowed to acclimate for 2 h before measurements were taken for 3 h. Resting VO_2 (ml/h/kg body weight) was calculated from the average of the values from the lowest 60 s sampling period for each mouse. Because body mass differed greatly between the genotypes it was critical to statistically control for effects of body mass (mostly fatness) on energy expenditure using analysis of covariance (ANCOVA) which tests for differences in the categorical variable (genotype) after accounting for the covariate (body mass). Mass specific O_2 consumption and body mass were $\log_{10}$ transformed to correct for allometric scaling before testing for genotype effects by ANCOVA.

To estimate the absorption of nutrients from food, chow and WT and *rgs2*$^{-/-}$ mouse fecal samples were collected, dried and sent for gross energy analysis (Wildlife Habitat and Nutrition Lab, Washington State University). Caloric intake was estimated as the difference between the caloric contents of chow ingested and feces excreted.

2.8. Adaptive thermogenesis

Six to eight-week-old male mice were housed at thermoneutrality (28 °C) for three days. Core body temperature was measured using a rectal temperature probe (Harvard Apparatus, Holliston, MA) before mice were transferred to individual cages at 4 °C and core temperature assessed every hour. After 7 h mice were sacrificed and interscapular brown adipose (BAT) and gonadal white adipose tissues (WAT) were rapidly collected and frozen.

2.9. Glucose tolerance and insulin sensitivity tests

Mice were fasted for 6 h (glucose), or 2 h (insulin) to achieve baseline blood glucose levels. Following the fast, blood glucose was measured from a tail snip (Freestyle mini blood glucose monitoring system, Abbott laboratories, Quebec, Canada). Mice were injected with a bolus of glucose (1 mg/kg i.p.) or insulin (0.4 IU/kg s.c., Novolin®ge Toronto, Novo Nordisk, Mississauga, Canada) and blood glucose levels determined after 15, 30, 60 and 120 min.

2.10. Real time RT-PCR

Total RNA was extracted from frozen tissues using Trizol Reagent (Invitrogen Life Techologies). RNA from WAT and BAT was further purified on RNeasy mini columns (Qiagen, Mississauga, ON, Canada). 200 ng total RNA was reverse transcribed with a high capacity reverse transcription kit (Applied Biosystems, Streetsville, ON, Canada). Real-time PCR was performed on an ABI Prism 7900HT sequence detection system (Applied Biosystems, Streetsville, ON, Canada) using Taqman gene expression assays from Applied Biosystems (Supplementary Table S1) and normalized to levels of the endogenous control GAPDH or β-actin.

2.11. Western blotting

Anti-UCP-1 (1:1000 dilution) and anti-β-tubulin (loading control; 1:2000, both from Abcam Inc, Cambridge, MA) were used in BAT lysates prepared in RIPA buffer (Cell Signaling Technology, Inc, MA). Anti-phospho-glycogen synthase (S641) (1:1000 dilution) and anti-glycogen synthase (1:1000 dilution both from Cell Signaling Technology, Inc.) were used to examine the phosphorylation state of hepatocyte glycogen synthase. Western blotting was performed as described previously [9]. Blots were quantified by densitometry analysis using AlphaEaseFC software (FluorChem 8000 Advanced Chemiluminescence and Visible Light Imaging, Alpha Innotech Corporation).

2.12. Statistical analysis

Data are presented as means ± SEM. Data were analysed using one way ANOVA, ANCOVA, linear regression or Student's *t*-test. Differences were considered significant at $P<0.05$.

3. Results

3.1. Resistance to age-related weight gain in *rgs2*$^{-/-}$ mice

The main finding of this study is that *rgs2*$^{-/-}$ mice are resistant to the age-related weight gain of wild-type C57BL/6 mice (Fig. 1). To investigate the possible emergence of latent phenotypes, we kept a group of wild-type and *rgs2*$^{-/-}$ mice to the age of 21–24 months old. The C57BL/6 genetic background of the *rgs2*$^{-/-}$ strain is regarded as 'obesity prone' [32], and these animals ordinarily are susceptible to age- and diet-induced obesity [33]. Older *rgs2*$^{-/-}$ mice were clearly smaller than their wild-type C57BL/6 counterparts (Fig. 1A), and correspondingly 21–24 month old *rgs2*$^{-/-}$ mice of both sexes were significantly lighter than age-matched wild-type controls (Fig. 1B). Heterozygous *rgs2*$^{+/-}$ animals were indistinguishable from wild-type mice with respect to body weight (data not shown). A discrepancy in body weight between wild-type and *rgs2*$^{-/-}$ mice became evident as early as 4 weeks of age and increased through the first year of life in both males (Fig. 1C) and females (data not shown). Changes in weight were also recorded throughout the second year of life in males, and *rgs2*$^{-/-}$ animals remained relatively constant over this period whereas wild-type mice gained weight intermittently until about 1.5 years of age (at which point the difference between strains was maximal), and this was followed by a gradual diminution in size for the remainder of the experimental period (Fig. 1C).

3.2. Body fat mass and serum biochemistry in *rgs2*$^{-/-}$ mice

We hypothesized that the difference in body weight between the *rgs2*$^{-/-}$ and wild-type mice was likely to be due to reduced adipose tissue deposits in the *rgs2*$^{-/-}$ mice. To examine this we weighed gonadal (i.e., epididymal or uterine) white adipose tissues (WAT) upon sacrifice of the aged mice. The lower body weights of *rgs2*$^{-/-}$ mice corresponded to gonadal white adipose tissue weights that were reduced by about 70% in male mice and 85% in female mice compared to wild-type controls (Fig. 2A), suggesting that strain-related differences in body weight reflect differences in adiposity. Consistent with smaller fat stores, serum-free fatty acids and triglycerides were significantly lower in serum from male *rgs2*$^{-/-}$ mice compared to wild-type (Fig. 2B and C). Leptin was also reduced in the RGS2 knockouts (Fig. 2D). Cholesterol levels were similar between wild-type and *rgs2*$^{-/-}$ mice (75.3 ± 3.6 and 70.7 ± 6.7 mg/dl wild-type and *rgs2*$^{-/-}$ respectively, $n \geq 9$, $P = 0.512$). Lipid levels in female mice were similar between wild-type and *rgs2*$^{-/-}$ (data not shown). Thus overall adiposity is reduced in older *rgs2*$^{-/-}$ mice compared to wild-type. In addition, histological analysis showed cross-sectional areas of individual white adipocytes from *rgs2*$^{-/-}$ mice to be smaller by about

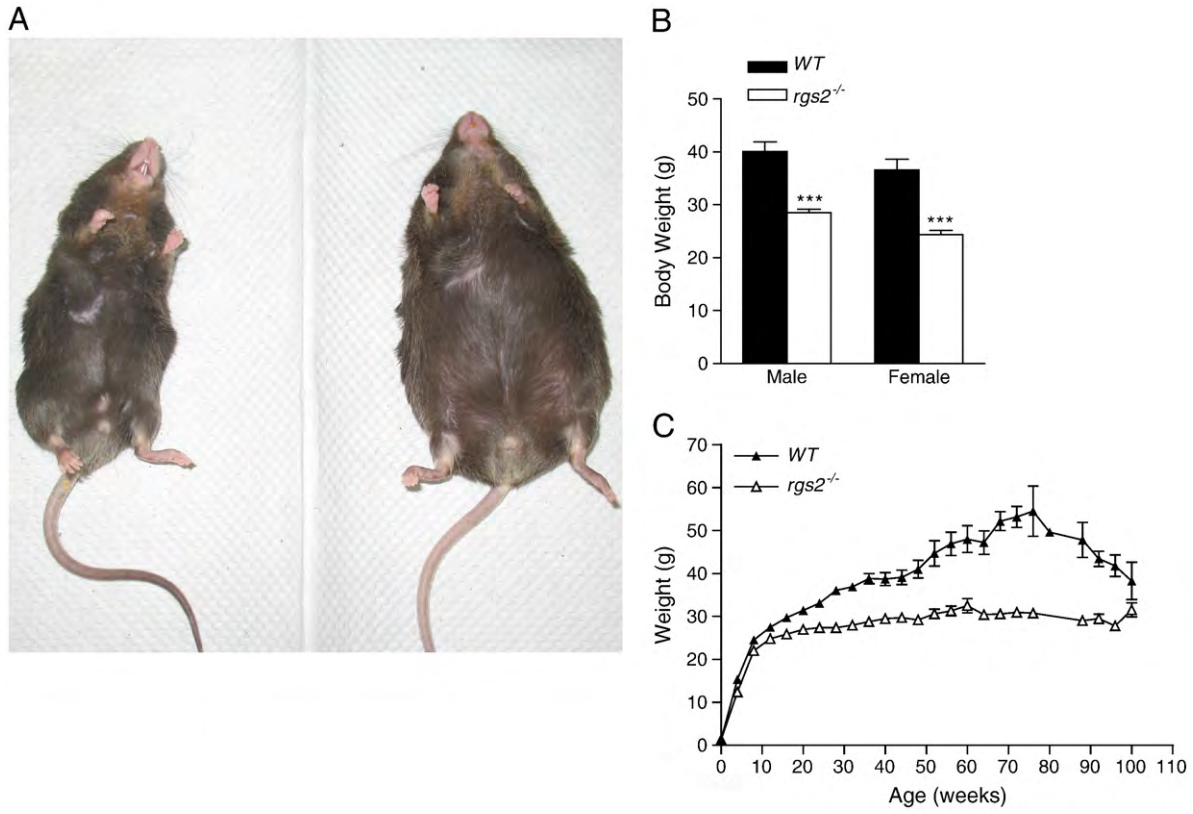

Fig. 1. Lower body weight of aged (21–24 month) *rgs2*$^{-/-}$ mice. (A) Male *rgs2*$^{-/-}$ (left) and wild-type (right) mice at 23 months of age. (B) Body weight in male and female *rgs2*$^{-/-}$ is significantly lower than WT at 21–24 months old. Data represent means ± S.E.M., n ≥ 5 (female); n ≥ 9 (male). ***$p<0.001$ *t*-test comparing WT and *rgs2*$^{-/-}$. (C) Growth curve of male wild-type and *rgs2*$^{-/-}$ mice. Data represent means ± S.E.M., n ≥ .4.

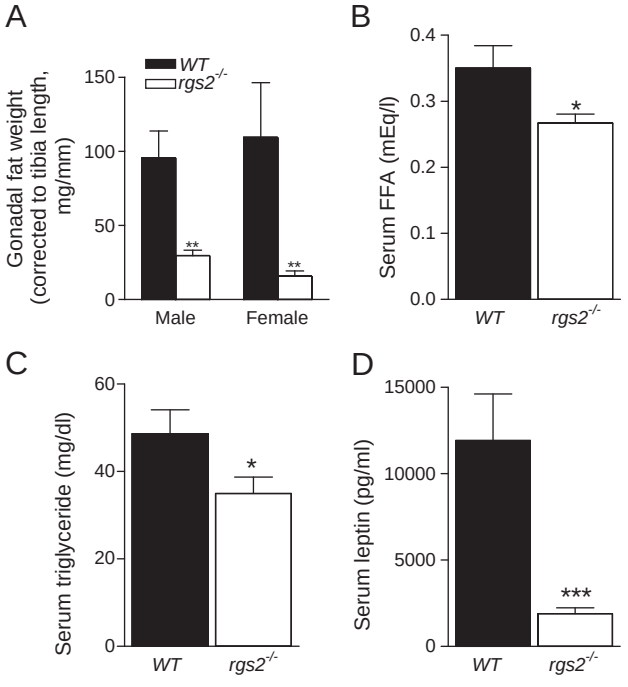

Fig. 2. Reduced adiposity of aged (21–24 month) *rgs2*$^{-/-}$ mice. (A) Gonadal fat weight of WT and *rgs2*$^{-/-}$ mice upon sacrifice (n ≥ 4 female; n ≥ 6 male). (B) Free fatty acids (FFA), (C) triglyceride and (D) leptin levels in serum from male mice taken at sacrifice n ≥ 9, *$p<0.05$, **$p<0.01$, ***$p<0.001$ *t*-test comparing WT and *rgs2*$^{-/-}$. Data represent means ± S.E.M.

45% in males and 30% in females compared to cells from wild-type mice (Fig. 3). It follows that reduced fat storage in individual adipose cells may contribute to the smaller fat deposits and lower body weight found in these animals. There was no obvious difference in the appearance of brown adipose tissue, liver or pancreatic islets from 21 to 24 month old wild-type and *rgs2*$^{-/-}$ mice (data not shown).

To better understand the lean *rgs2*$^{-/-}$ phenotype, we also investigated younger adult male animals. For these studies an age of 34 weeks was chosen, as at this point weights were clearly different between strains but still diverging (Fig. 1C). We examined differences in body composition in living animals by performing magnetic resonance scans of 34 week old mice. This showed that the fat mass of the *rgs2*$^{-/-}$ mice was reduced by about 70% compared to wild-type controls (Table 1). Lean mass was also significantly lower but only by about 10%, implying that the ~10 g body weight difference between strains was due primarily to differences in fat mass (Table 1). This was confirmed by comparing weights of individual fat pads (epididymal, inguinal, retroperitoneal and brown fat, (Table 1) from wild-type and *rgs2*$^{-/-}$ mice. A comparison between the data in Fig. 2 and Table 1 suggests that the difference in gonadal fat pad weight evident in older animals is established relatively early in life. Also, in both strains there appears to be an age-dependent redistribution of regional fat stores (compare Fig. 2A with Table 1), as older *rgs2*$^{-/-}$ and wild-type mice had smaller epididymal deposits than 34 week old mice of the same respective strain in spite of similar overall body weights (see Fig. 1C). In contrast to white fat deposits, heart and kidney weight were not different between wild-type and *rgs2*$^{-/-}$ mice. Liver weight was significantly lower in *rgs2*$^{-/-}$ mice, which may be due to lower levels of fat storage in the livers of *rgs2*$^{-/-}$ mice.

Serum biochemistry in 34 week old mice (Table 1) showed comparable strain-related differences to those observed in aged animals (Fig. 2B, C and D). Serum triglycerides and cholesterol at

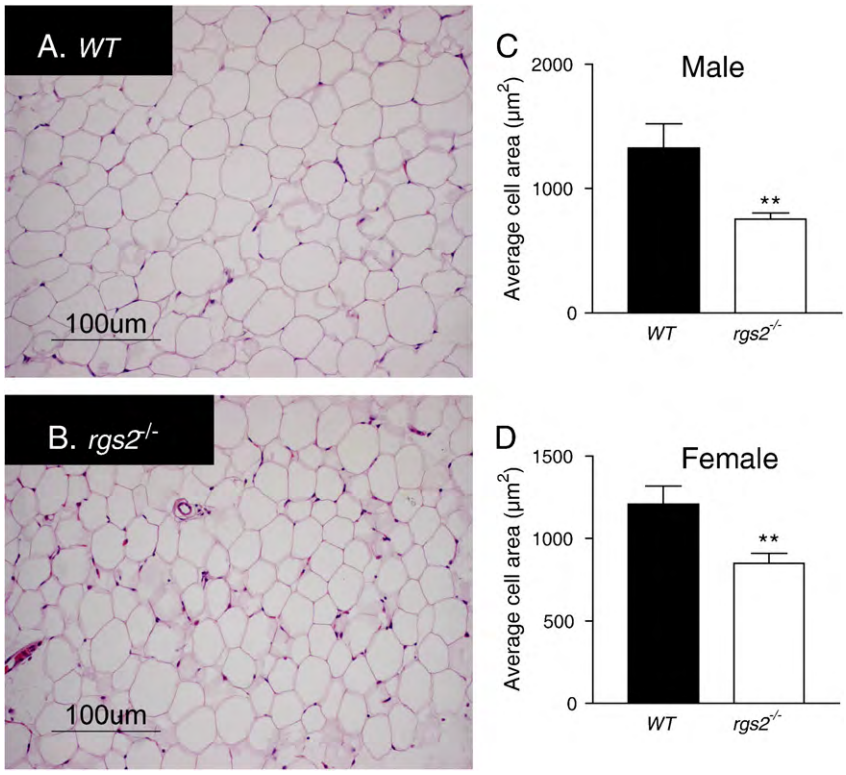

Fig. 3. WAT histology in aged (21–24 month) *rgs2*$^{-/-}$ and WT male mice. Hematoxylin and eosin stained sections of white adipose tissue (A) and (B). Male (C) and female (D) white adipose cell size was quantified by determining the average cell area at 20× magnification using a Northern Eclipse image analysis system. Data represent means ± S.E.M., n ≥ 5, $^{**}p<0.01$ *t*-test comparing WT and *rgs2*$^{-/-}$.

34 weeks were significantly lower in *rgs2*$^{-/-}$ compared to wild-type mice, and this was accompanied by reduced insulin, leptin and adiponectin, with no change in fasting glucose levels (Table 1). Most other cardiovascular and adipogenic biomarkers were unchanged (Supplementary Table 2). Together these data suggest that reduced adiposity in *rgs2*$^{-/-}$ mice is established by early-to-mid adulthood. A further analysis of the serum biomarker data using analysis of covariance (ANCOVA), which controls for effects of body mass (mostly fatness) by testing for differences in the categorical variable (genotype) after accounting for the covariate (body mass), suggested that the observed strain-related differences in leptin and insulin levels were due largely to the difference in fat mass, consistent with the physiological correlation between these hormones and adipose tissue [34].

Table 1
Body, fat, lean and organ weights, and serum biochemistry of 34 week old male mice.

		WT	*rgs2*$^{-/-}$
Body weight	(g)	35.6 ± 1.0	25.6 ± 0.4***
Fat weight	(g)	11.5 ± 0.7	3.4 ± 0.2***
Lean weight	(g)	22.6 ± 0.5	20.5 ± 0.2**
Organ weight corrected for tibia length			
Epididymal fatpad	(mg/mm)	143.5 ± 4.9	80.8 ± 5.8***
Inguinal fatpad	(mg/mm)	53.0 ± 5.0	14.7 ± 0.9***
Retroperitoneal fatpad	(mg/mm)	43.6 ± 3.2	9.0 ± 0.8***
Brown adipose tissue	(mg/mm)	8.3 ± 0.2	3.8 ± 0.5***
Kidney	(mg/mm)	11.0 ± 0.4	10.4 ± 0.5
Liver	(mg/mm)	65.6 ± 1.7	53.4 ± 1.7***
Heart	(mg/mm)	7.9 ± 0.3	7.3 ± 0.3
Free fatty acids	(mEq/l)	0.56 ± 0.04	0.48 ± 0.04
Triglycerides	(mg/dl)	79.7 ± 3.9	55.2 ± 3.7***
Cholesterol	(mg/dl)	134.3 ± 3.5	119.0 ± 5.9**
Glucose	(mmol/l)	14.1 ± 1.3	16.0 ± 1.4
Insulin	(pg/ml)	407.0 ± 44.8	155.6 ± 55.2**
Leptin	(ng/ml)	5.41 ± 0.82	0.59 ± 0.17***
Adiponectin	(μg/ml)	24.0 ± 1.5	17.9 ± 0.8**

Data represent means ± S.E.M., n ≥ 8. *** $P<0.0001$, ** $P<0.01$, *t*-test comparing WT and *rgs2*$^{-/-}$.

3.3. Altered gene expression in *rgs2*$^{-/-}$ mice

The loss of fat stores in the absence of RGS2 protein presumably reflects the perturbation of RGS2-dependent cellular processes. For example, RGS2 has been found to play a key role in an in vitro model of adipocyte differentiation, wherein its expression is induced by the treatment of pre-adipocytes by differentiating factors, and this increase in turn appears to promote the transition to a mature adipocyte phenotype [16,23]. It follows that adipocyte differentiation may be abnormal in *rgs2*$^{-/-}$ mice. To examine this possibility, undifferentiated pre-adipocytes were isolated from both strains and compared for the expression of four key genes that are important in adipocyte maturation (Fig. 4). In cells isolated from *rgs2*$^{-/-}$ mice, the basal levels of PPARγ, CEBP, and Lpl were significantly lower than controls. Upon treatment with differentiation stimuli, the expression of Lpl mRNA increased in WT cells but not *rgs2*$^{-/-}$ cells. These results suggest that adipocyte differentiation is altered in *rgs2*$^{-/-}$ animals, and this may contribute to the observed lean phenotype.

We also examined the mRNA expression in tissues of genes that contribute to fat metabolism. SREBP-1 was about 15% lower in WAT from *rgs2*$^{-/-}$ mice compared to wild-type, reinforcing the idea that adipocyte differentiation may be impaired in these mice (Fig. 5A). In brown adipose tissue from young adult mice (12 weeks of age), UCP1 (uncoupling protein 1) was found to be significantly downregulated in *rgs2*$^{-/-}$ animals at both the protein and mRNA levels (Fig. 5B); lower levels of this protein would be expected to decrease fatty acid oxidation and thus would be expected to increase fat accumulation, suggesting that the observed change in UCP1 might be a compensatory effect. In addition, we measured the mRNA levels in skeletal muscle, cardiac muscle, and liver of various other genes involved in mitochondrial biogenesis, electron transport, oxidative phosphorylation and β-oxidation

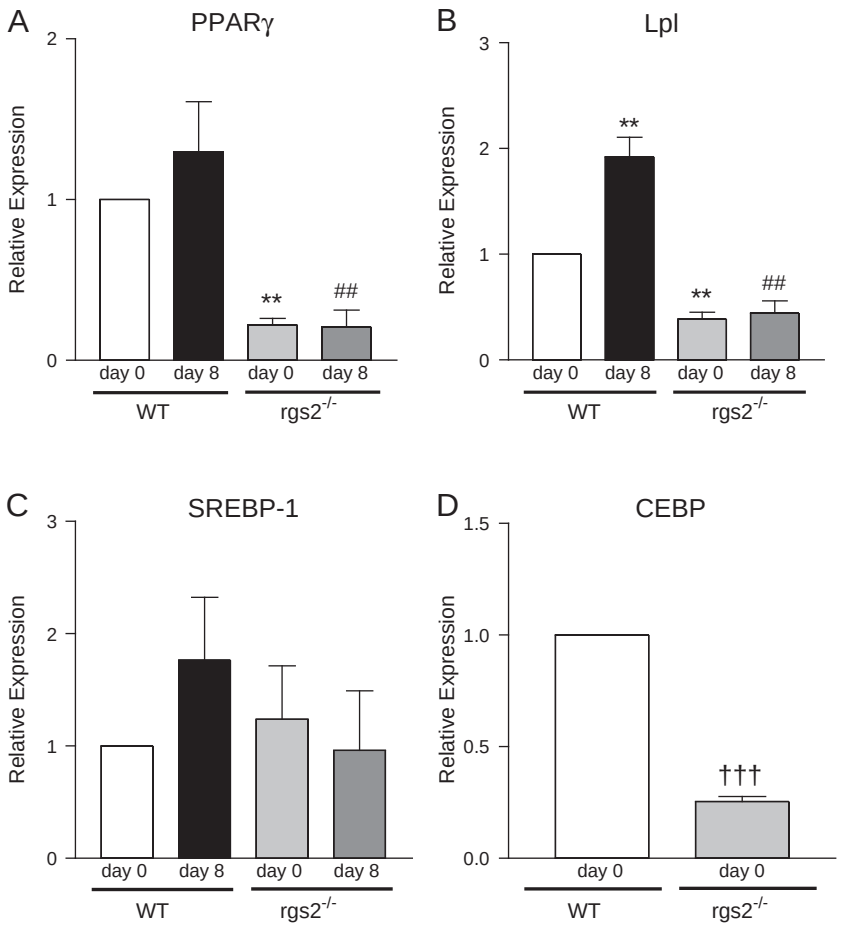

Fig. 4. In vitro adipogenesis. Pre-adipocytes were isolated from 3 months old WT and *rgs2*$^{-/-}$ male mice. The cells were treated with differentiation cocktail (dexamethasone, IBMX and insulin) 2 days post confluence. Expression of adipogenesis markers were measured before and 8 days after stimulation by real-time PCR. Data represent means ± SEM of 3 independent experiments with duplicate samples. $^{**}p<0.01$ compared to day 0 of WT. ## $p<0.01$ compared to day 8 of WT One way ANOVA with Tukey's post-test, †††$p<0.001$ *t*-test compared to day 0 of WT.

(including NADH dehydrogenase (ubiquinone) flavoprotein 1, Cytochrome C oxidase subunit 4, ATP synthase, Nuclear respiratory factor 1, Aminolevulinic acid synthase 1, Carnitine palmitoyltransferase 1 α, and Medium chain acyl-coenzyme A dehydrogenase). There was no difference in expression of these genes between wild-type and *rgs2*$^{-/-}$ animals in skeletal muscle, and little difference in liver and cardiac muscle (see Supplementary Fig. 1).

3.4. GPCR signaling

RGS2 is best known for its ability to inhibit GPCR signals, in particular those mediated via Gαq and Gαs proteins [1]. In the liver, signaling through the G protein coupled glucagon and β-adrenergic receptors leads to protein kinase A (PKA)-mediated phosphorylation of key enzymes which regulate glycogen synthesis, glycogen breakdown, and gluconeogenesis [35]. Thus we hypothesized that deregulated signaling through these receptors might lead to metabolic disturbances and differences in body weight. In order to test this we examined the effects of the β-adrenergic agonist isoproterenol (Iso) as well as glucagon on cAMP accumulation in hepatocytes. We found that while the Gs-coupled increase in cAMP was identical between hepatocytes from wild-type and *rgs2*$^{-/-}$ in response to glucagon, isoproterenol-induced cAMP accumulation was significantly greater in hepatocytes from *rgs2*$^{-/-}$ livers, with no change in pEC_{50} (Fig. 6A, B). Thus signaling through β-adrenergic receptors appears to be enhanced in livers from *rgs2*$^{-/-}$ mice.

To determine the possible downstream consequences of increased Gs-mediated signaling on carbohydrate metabolism in hepatocytes, we also examined the effects of isoproterenol and glucagon on glucose/glycogen balance by measuring glucose production and also the phosphorylation state of glycogen synthase, which converts glucose to glycogen. The latter is phosphorylated by cAMP-stimulated protein kinase A, a post-translational modification which decreases its activity [36]. In unstimulated cells, immunoblots based on a phospho-specific glycogen synthase antibody were similar in both strains relative to the signal produced by a pan-glycogen synthase antibody, and total levels of the enzyme also appeared equivalent in the cells derived from WT and *rgs2*$^{-/-}$ livers. In contrast to these similarities between strains under basal conditions, both isoproterenol and glucagon significantly enhanced the phosphorylation of glycogen synthase in *rgs2*$^{-/-}$ mice, whereas their effects on the WT mice were relatively modest (Fig. 6C–E). Similar strain-related differences were found with respect to hepatocyte glucose production, as basal activities were indistinguishable whereas isoproterenol and glucagon each significantly increased glucose production in the *rgs2*$^{-/-}$ mice, but exhibited little or no measurable effect in their WT littermates (Fig. 6F, G).

The observed increases in β-adrenergic and glucagon receptor signaling in *rgs2*$^{-/-}$ mice suggest that altered glucose/glycogen balance due to increased cAMP levels may be a contributing factor to the reduced fat stores and increased insulin sensitivity (see following section) in the *rgs2*$^{-/-}$ mice. An increase in Gs-induced adenylyl cyclase activity is consistent with the established effects of

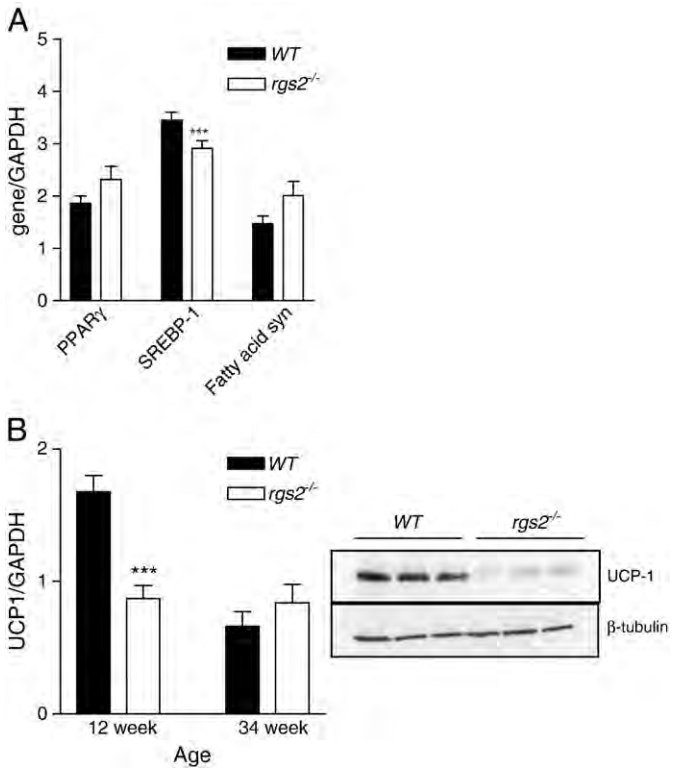

Fig. 5. Gene expression. (A) Expression of white adipose tissue adipogenic genes in tissues from 34 week old WT and *rgs2*$^{-/-}$ male mice. WT and *rgs2*$^{-/-}$. Abbreviations: PPARγ (Peroxisome proliferator-activated receptor γ), SREBP-1 (Sterol regulatory element binding factor 1). (B) Gene and protein expression of UCP1 in animals at room temperature assessed by real-time PCR and Western blotting. Data represent means ± S.E.M., n ≥ 8, ***$p < 0.0001$ *t*-test comparing WT and *rgs2*$^{-/-}$.

RGS2 on signaling, but it is conceivable that increased cellular cAMP levels in *rgs2*$^{-/-}$ hepatocytes could also reflect altered phosphodiesterase (PDE) activity [37]. To minimize such potential effects, all assays of β-adrenergic and glucagon receptor signaling were carried out in the presence of the general PDE inhibitor IBMX. We also examined mRNA expression levels of two relevant isoforms and found no significant strain-related differences under either basal or agonist-stimulated conditions (1-hour treatment with isoproterenol or glucagon) in either PDE4D, which selectively acts on cAMP and is ubiquitously expressed [38], or PDE3B, a non-selective cAMP and cGMP phosphodiesterase that is highly expressed in hepatocytes [39] (data not shown). Thus the observed differences in Gs-mediated signaling between WT and *rgs2*$^{-/-}$ animals seem to be independent of any strain-related differences in PDE.

In white adipose tissue, lipolysis is promoted by β-adrenergic and other Gs-coupled as well as Gq-coupled receptors, whereas Gi-mediated signals tend to inhibit lipolysis [35]. While increased lipolytic signaling of Gs- and Gq-coupled receptors (due to the absence of RGS2) is another possible mechanism to explain the smaller fat stores in the *rgs2*$^{-/-}$ mice, we could not detect any strain-related difference in isoproterenol- or phenylephrine-promoted glycerol release in freshly isolated white adipose tissue (Supplementary Fig. 2). This suggests that basal RGS2 levels are not high enough to affect adrenergic receptor signaling in these cells, although such a mechanism could conceivably be engaged under conditions where RGS2 is upregulated [9].

3.5. Metabolic studies on *rgs2*$^{-/-}$ mice

3.5.1. Food intake and metabolic rate

Differences in body weight can be due to either altered energy intake or altered energy expenditure. We studied energy (food) intake over time and found that the *rgs2*$^{-/-}$ mice are slightly hyperphagic (Fig. 7A). Dry weight and caloric content of feces were not different between strains (data not shown), indicating that there was no deficiency in the ability of *rgs2*$^{-/-}$ mice to absorb nutrients from food. Resting mass specific O_2 consumption was affected by body mass (ANCOVA, $P = 0.008$) and after correcting for body mass we found that surprisingly *rgs2*$^{-/-}$ had a significantly lower metabolic rate than wild-type (0.66 ± 0.07, 1.00 ± 0.07 least squares mean log mass specific metabolic rate, *rgs2*$^{-/-}$ and wild-type respectively, $P = 0.009$; Fig. 7B). Thus the mice have reduced body weight despite eating more and expending less energy at rest.

3.5.2. Thermogenesis

The expenditure of energy as heat to maintain body temperature is under the control of β-adrenergic receptors in brown adipose tissue (BAT). To test whether *rgs2*$^{-/-}$ mice have increased thermogenic capacity we subjected the mice to a cold challenge (4 °C, 7 h). These experiments found that the mice had neither a difference in basal body temperature nor response to cold challenge (Fig. 7C). Furthermore, the mice had similar changes in BAT expression of thermogenic genes in response to cold challenge. Notably though, wild-type RGS2 expression was significantly reduced in response to cold challenge (Fig. 7D), which would tend to suggest that a normal function of RGS2 may be to impede thermogenesis. Overall it appears that the *rgs2*$^{-/-}$ mice do not expend excess energy through acute thermogenesis, however.

3.5.3. Insulin sensitivity

Lean mice often have altered glucose metabolism and insulin sensitivity. Notwithstanding the observed increases in hepatocyte responses to isoproterenol and glucagon (Fig. 6), the *rgs2*$^{-/-}$ mice in this study were found to have normal fasting serum glucose levels and also reduced serum insulin levels (Table 1), suggesting increased insulin sensitivity. In support of this, the *rgs2*$^{-/-}$ mice were able to clear a bolus of glucose from their bloodstream significantly faster than wild-type in glucose tolerance tests (Fig. 8A). Furthermore, in insulin sensitivity tests *rgs2*$^{-/-}$ mice maintained lower blood glucose levels than wild-type following insulin bolus (Fig. 8B). These data are consistent with the serum data and suggest that insulin sensitivity may be a cause or a result of the lean phenotype of *rgs2*$^{-/-}$ mice.

3.6. High fat diet

Since the *rgs2*$^{-/-}$ mice are resistant to age-related weight gain, we hypothesized that they might also be resistant to high fat diet-induced obesity. To test this, the mice were fed a high fat diet for 25 weeks. Surprisingly the *rgs2*$^{-/-}$ mice were not resistant to gaining weight on a high fat diet (Fig. 9A), Furthermore, the differences in lean body weight, insulin sensitivity and serum leptin levels between wild-type and *rgs2*$^{-/-}$ were abolished after an extended time on the diet (Fig. 9B, C, D, and Supplementary Table 2). Fat mass was significantly different between wild-type and *rgs2*$^{-/-}$ mice but again this may be due to the difference in fat mass at the start of the diet. Interestingly, a high fat diet produced changes in RGS2 expression in wild-type animals in an organ specific manner: In WAT and liver, RGS2 expression was higher in mice fed a high fat diet compared to those fed regular diet, suggesting that RGS2 may promote fat storage in these tissues. In contrast in cardiac muscle RGS2 expression was lower in mice fed a high fat diet (Fig. 9E).

4. Discussion

The present study describes a previously unreported phenotype of the *rgs2*$^{-/-}$ mice in which they are resistant to normal, age-related weight gain. Lower body weight is accompanied by increased insulin sensitivity, smaller adipose tissue deposits, smaller adipocytes and

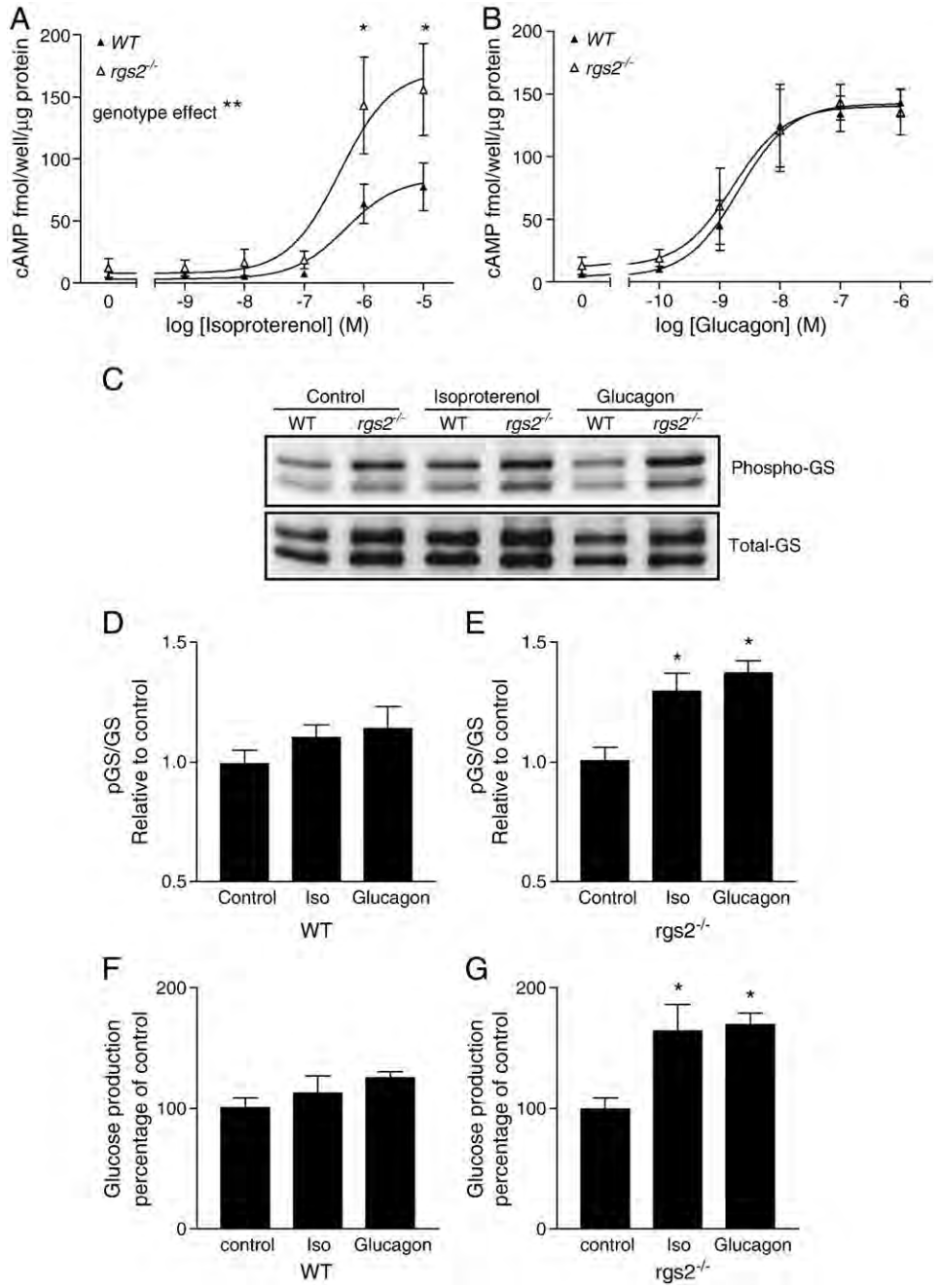

Fig. 6. Receptor signaling. Isoproterenol and glucagon stimulated cAMP accumulation (A,B), glycogen synthase phosphorylation (C–E) and glucose production (F,G) in primary hepatocytes isolated from WT and *rgs2*$^{-/-}$ mice. Data represent means ± S.E.M., $n = 3$ experiments performed in duplicate. Values of glycogen synthase phosphorylation shown were calculated from densitometric scans of immunoblots (C) of phosphorylated glycogen synthase relative to total glycogen synthase and then normalized to the average values of WT and *rgs2*$^{-/-}$ untreated samples (D, E). Glucose production was normalized to the average values of WT and *rgs2*$^{-/-}$ untreated samples (F, G). Genotype effect: $^{**}p < 0.01$ two-way ANOVA; significant differences at specific times: $^{*}p < 0.05$ two-way ANOVA with Bonferroni post-test.

lower levels of serum lipids. This phenotype is evident in mice as young as 4 weeks old, but does not prevent the mice from reaching a healthy adult weight, suggesting that other factors such as bone and muscle formation are minimally affected. These data suggest a novel role for RGS2 in energy metabolism and body fat regulation which may involve multiple mechanisms, including the promotion of adipocyte differentiation and also the negative regulation of GPCR signaling.

RGS2 has been implicated in the differentiation of adipocytes in vitro [23], and the present results suggest that this is reflected in vivo. Although adipocytes were smaller in aged *rgs2*$^{-/-}$ mice (Fig. 2), this decrease in cell size was not consistent in scale with the large difference in adipose tissue size, implying a reduction in the number of fat cells. It follows that adipocyte formation or survival may be impaired in the *rgs2*$^{-/-}$ mice. Indeed, RGS2 has been shown in other studies to be involved in adipocyte differentiation and thus impaired adipocyte differentiation may contribute to the lean phenotype observed herein. The *rgs2*$^{-/-}$ mice have lower WAT expression of the adipogenic transcription factor SREBP-1c than wild-type, accompanied by lower UCP1 expression in BAT in young (12 week) *rgs2*$^{-/-}$ mice compared to the wild-type, which suggests that adipocyte differentiation and/or function may be impaired in the *rgs2*$^{-/-}$ mice. Moreover, pre-adipocytes isolated from *rgs2*$^{-/-}$ mice showed reduced mRNA levels of three genes (out of four tested) known to

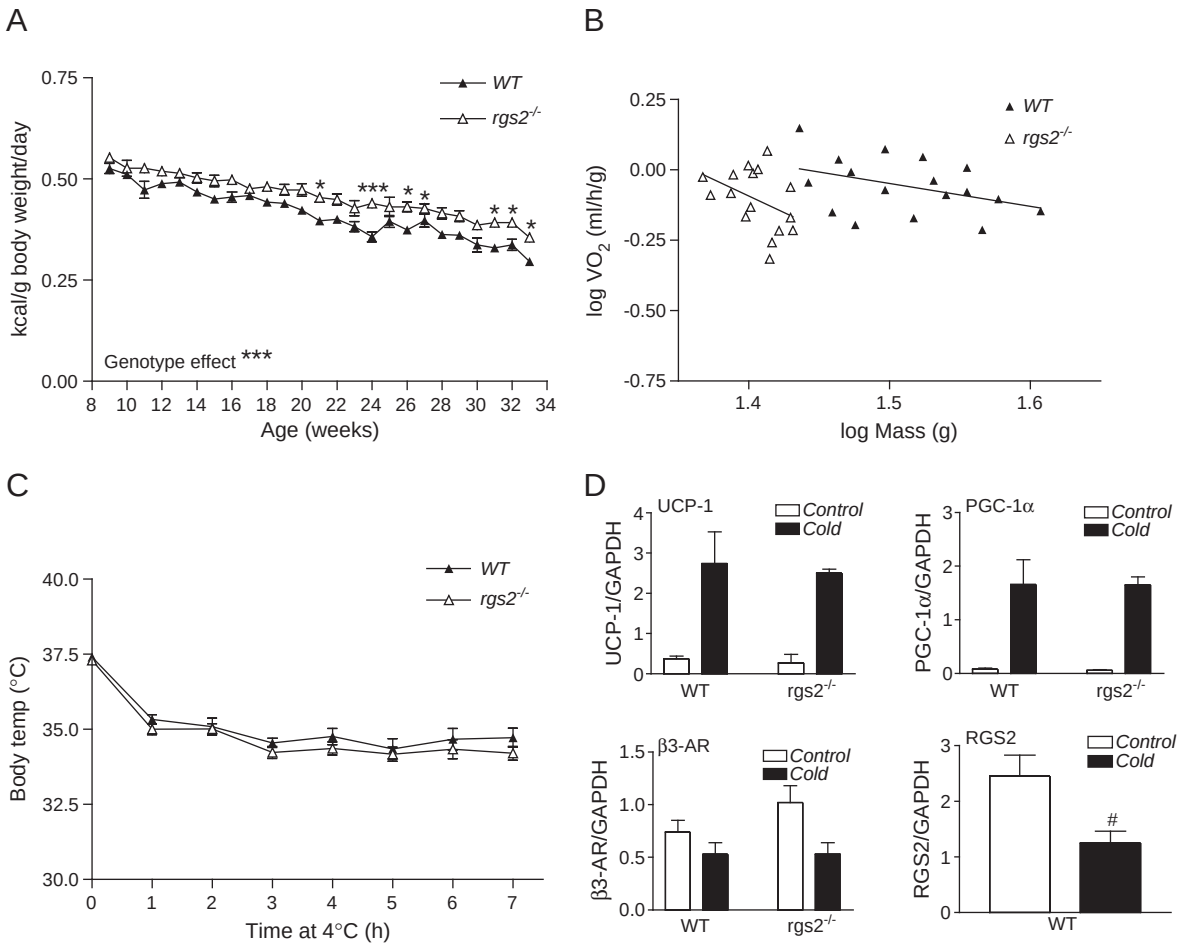

Fig. 7. Food intake, energy expenditure and adaptive thermogenesis in WT and *rgs2*$^{-/-}$ male mice. (A) Food intake per mouse over 25 weeks normalized to body weight. (B) Resting oxygen consumption measured by indirect calorimetry during a 3-hr period. (C) Adaptive thermogenic response to 7 h cold (4 °C) challenge, (D) gene expression of RGS2 and thermogenic proteins in interscapular BAT assessed by real-time PCR. Data represent means ± S.E.M., n ≥ 8, genotype effect: ***p<0.0001 two-way ANOVA; significant differences at specific times: *p<0.05, ***p<0.0001 two-way ANOVA with Bonferroni post-test; #p<0.05, t-test comparing cold and control treated animals.

be important for adipocyte maturation. Overall, our results suggest that in the absence of RGS2 adipocyte differentiation is impaired, while the reduced size of these cells could be due to either altered lipid handling or reduced free fatty acid availability.

The most well established mechanism of action of RGS2 is negative modulation of signaling through GPCRs, particularly ones that activate Gq- and/or Gs. Thus it follows that the mechanism by which phenotypic abnormalities are occurring in mice without RGS2 would likely involve enhanced GPCR signaling. In concert with the present findings, a number of recently described mouse models have demonstrated an emerging role for G protein signaling in energy homeostasis and the pathogenesis of obesity: Mice in which Gαq is conditionally inactivated in adipose tissue have increased body weight and fat mass compared to wild-type [40]; Mice with enhanced PKA activity in adipose tissue display a lean phenotype with reduced serum leptin, adipocyte triglyceride storage and increased metabolic rate and body temperature [41]; RGS-insensitive Gαi2^{G184S} mice (genomic "knock-in" of Gαi2 with a G184S mutation which blocks RGS protein binding) are lean, have increased insulin sensitivity, and are resistant to diet-induced obesity [42]; AGS3 (activator of G protein signaling) knockout mice have lower body weight and increased energy expenditure compared to wild-type [43]; finally both RGS4 and RGS5 knockout mice weigh less than their wild-type counterparts [44,45]. The body uses a wide variety of G protein-mediated signaling pathways to regulate the intake, expenditure, and storage of caloric energy, and thus the specific mechanisms underlying the lean phenotypes associated with altered signaling may vary from one animal model to the next. Given the known G protein selectively of RGS2, it seems likely that enhanced signaling through Gq- and Gs-mediated pathways might contribute to the lean phenotype observed herein.

GPCRs play a significant role in controlling metabolic processes in the liver, where Gs-coupled β-adrenergic and glucagon receptors are known to promote glycogen breakdown, and gluconeogenesis, and also inhibit glycogen and fatty acid synthesis [35,46]. It is possible that increased stimulation of these pathways due to the loss of RGS2 could result in reduced energy stores. Here we show that activation of either β-AR or glucagon receptors induces a higher level of glycogen synthase phosphorylation as well as greater glucose production in isolated hepatocytes from *rgs2*$^{-/-}$ compared to WT mice. Thus it seems likely that the loss of RGS2 in the liver favors the production of glucose and disfavors the production of glycogen and fat, thereby contributing to the observed lean *rgs2*$^{-/-}$ phenotype.

In contrast to the similar changes in downstream signaling in *rgs2*$^{-/-}$ hepatocytes found with isoproterenol and glucagon, signaling at the level of the second messenger cAMP was increased in knockout cells upon β-adrenergic but not glucagon receptor activation. This finding is important since previous studies have demonstrated receptor selectivity for other RGS proteins [47,48] but to our knowledge this is the first example of receptor selectivity of endogenous RGS2. We have previously shown that RGS2 is able to bind to the β2-adrenergic receptor [49], and it is possible that this interaction may facilitate modulation of receptor activity by RGS2. Still, the enhancement of downstream glucagon

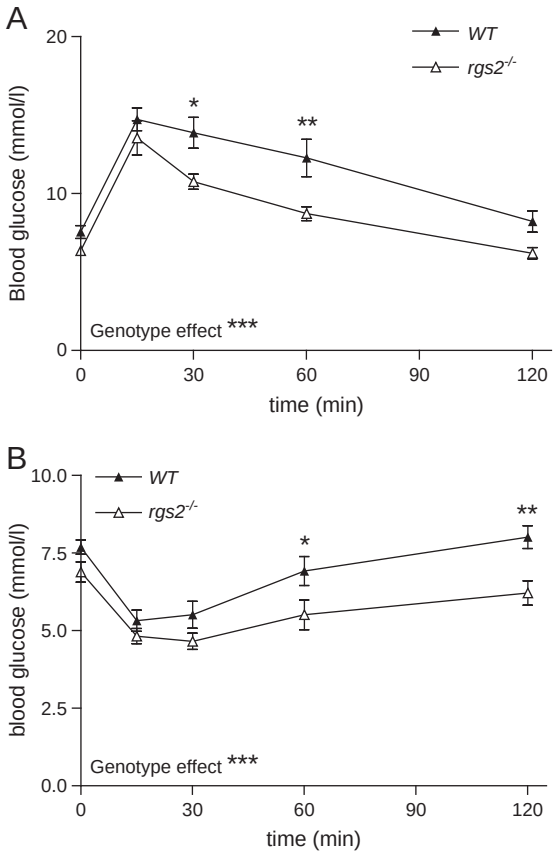

Fig. 8. Insulin sensitivity in *rgs2*$^{-/-}$ mice. (A) Glucose tolerance test: male mice were fasted for 6 h before i.p. injection of glucose (1 mg/kg). (B) Insulin sensitivity test: male mice were fasted for 2 hr before s.c. injection of insulin (0.4 IU/kg). Data represent means ± S.E.M., n ≥ 9, genotype effect: ***p<0.0001 two-way ANOVA; significant differences at specific times: *p<0.05, **p<0.01 two-way ANOVA with Bonferroni post-test.

signaling (Fig. 6C–G) was unexpected in light of its similar effects on cAMP levels in WT and *rgs2*$^{-/-}$ hepatocytes (Fig. 6B). This seeming inconsistency can potentially be explained, however, by taking into account the relatively long duration of the glycogen synthase phosphorylation and glucose release assays (60 min) versus the cAMP assay (1.5 min), and the tendency of RGS2 itself to be rapidly upregulated by increased cAMP [10,50]. In an earlier comparison between untreated WT and *rgs2*$^{-/-}$ osteoblasts, we were unable to detect any substantive strain-related differences in intracellular cAMP or Ca^{2+} respectively in response to PTH or purinergic receptor agonists, however the pretreatment of cells with agents known to upregulate RGS2 revealed differences in signaling via both of these receptors [9]. Therefore, we hypothesize that the rapid agonist-induced upregulation of RGS2 in WT but not *rgs2*$^{-/-}$ hepatocytes may have unmasked a strain-related difference in glucagon signaling within the longer time frame of the glucose production and glycogen synthase phosphorylation assays but that this would not have occurred within the comparatively brief incubation period of the cAMP assay.

The sympathetic nervous system (SNS) is one of the most important regulators of energy balance and adiposity through fat and glycogen catabolism, activation of adaptive thermogenesis in BAT, and regulation of heat loss through modulation of peripheral vasoconstriction and piloerection [51,52]. In many mouse models of obesity the β-adrengergic system is dysfunctional and its abilities to stimulate lipolysis and thermogenesis are both impaired [52]. Since sympathetic tone and noradrenaline levels appear to be increased in *rgs2*$^{-/-}$ mice [30], we hypothesized that these changes might contribute to the observed lean phenotype. Surprisingly, adaptive thermogenesis is apparently unchanged in our mice. The concentration dependence of adrenergic receptor-stimulated lipolysis was also indistinguishable between strains, although presumably such activity could be increased in the knockout animals in vivo due to higher circulating catecholamine levels [30].

In spite of the fact that normal adaptive thermogenesis can take place in the absence of RGS2, we found RGS2 gene expression in BAT to be reduced by approximately 50% following cold exposure (4 °C, 7 h). This is in agreement with a recent study using microarray analysis which found RGS2 to be downregulated by ~90% in the BAT of rats following prolonged cold exposure (4 °C, 48 h) [53]. These findings suggest that downregulation of RGS2 upon cold exposure may enable increased GPCR signaling during adaptive thermogenesis. Compensatory genetic changes are known to occur in *rgs2*$^{-/-}$ mice in order to maintain cellular homeostasis [54]. Thus it is possible that *rgs2*$^{-/-}$ mice are able to utilize redundant or alternative cold-adaptive mechanisms to limit hypothermia under cold challenge.

Similar to other mouse models with lowered body weight, the *rgs2*$^{-/-}$ mice have heightened insulin sensitivity. An increase in insulin sensitivity would also be consistent with our observation of normal levels of serum glucose in *rgs2*$^{-/-}$ animals in spite of increased hepatocyte responsiveness to isoproterenol and glucagon. There is some prior evidence for a role for RGS2 in glucose uptake, as ET-1-stimulated translocation of the GLUT4 insulin-responsive glucose transporter in 3T3-L1 adipocytes is blocked by the overexpression of RGS2 [16]. A lean phenotype may also reflect increased fatty acid oxidation and we examined this in the *rgs2*$^{-/-}$ animals by determining gene expression of key enzymes involved in this process in liver, skeletal muscle and cardiac muscle. These experiments revealed little difference in the expression levels of the majority of these enzymes between wild-type and *rgs2*$^{-/-}$, however (Supplementary Fig. 1).

We hypothesized that since the mice have reduced fat stores they would be resistant to high fat diet-induced obesity. Surprisingly this was not the case and the *rgs2*$^{-/-}$ mice gained weight at a similar rate as wild-type on a high fat diet (42% calories from fat). Furthermore the mice became insulin resistant on the high fat diet to a similar degree as the wild-type. The reason the mice were not resistant to a high fat diet is not clear but may suggest that lipid metabolism is not impaired in these mice, but rather carbohydrate or protein metabolism is altered. Alternatively, the energetic imbalance and/or impaired adipocyte differentiation underlying the lean phenotype may be overcome when the mice are subjected to energy overload as with the high fat diet. Interestingly, we found that RGS2 mRNA expression was higher in the WAT (also recently demonstrated by Song et al. [55]) and liver of wild-type fed a high fat diet while it was significantly lower in cardiac muscle and unchanged in BAT. These changes in expression of RGS2 following a high fat diet suggest that it is important in regulating energy storage and/or expenditure although exactly how is currently unclear. Increased expression of RGS2 in the important fatty acid metabolizing organs WAT and liver following increased substrate availability (i.e. high fat diet) suggests a role for RGS2 in fatty acid metabolism.

While the present results clearly implicate RGS2 as being an important protein in lipid homeostasis and also point to several possible mechanistic underpinnings, there are key questions that remain to be answered. In particular, it is difficult to explain the lack of evidence for increased metabolic function in *rgs2*$^{-/-}$ mice in the absence of either decreased caloric consumption or absorption. A lean phenotype may be a result of altered energy balance, leading to defects in metabolic processes and energy storage. Energy balance is a function of energy in (caloric intake) vs. energy out (energy expenditure). Food intake was actually increased in the *rgs2*$^{-/-}$, especially in the older mice (see Fig. 7A). This may be due to increased appetite stemming from lower leptin levels. Alternatively hyperphagia is common in lean mice and suggests a higher metabolic demand

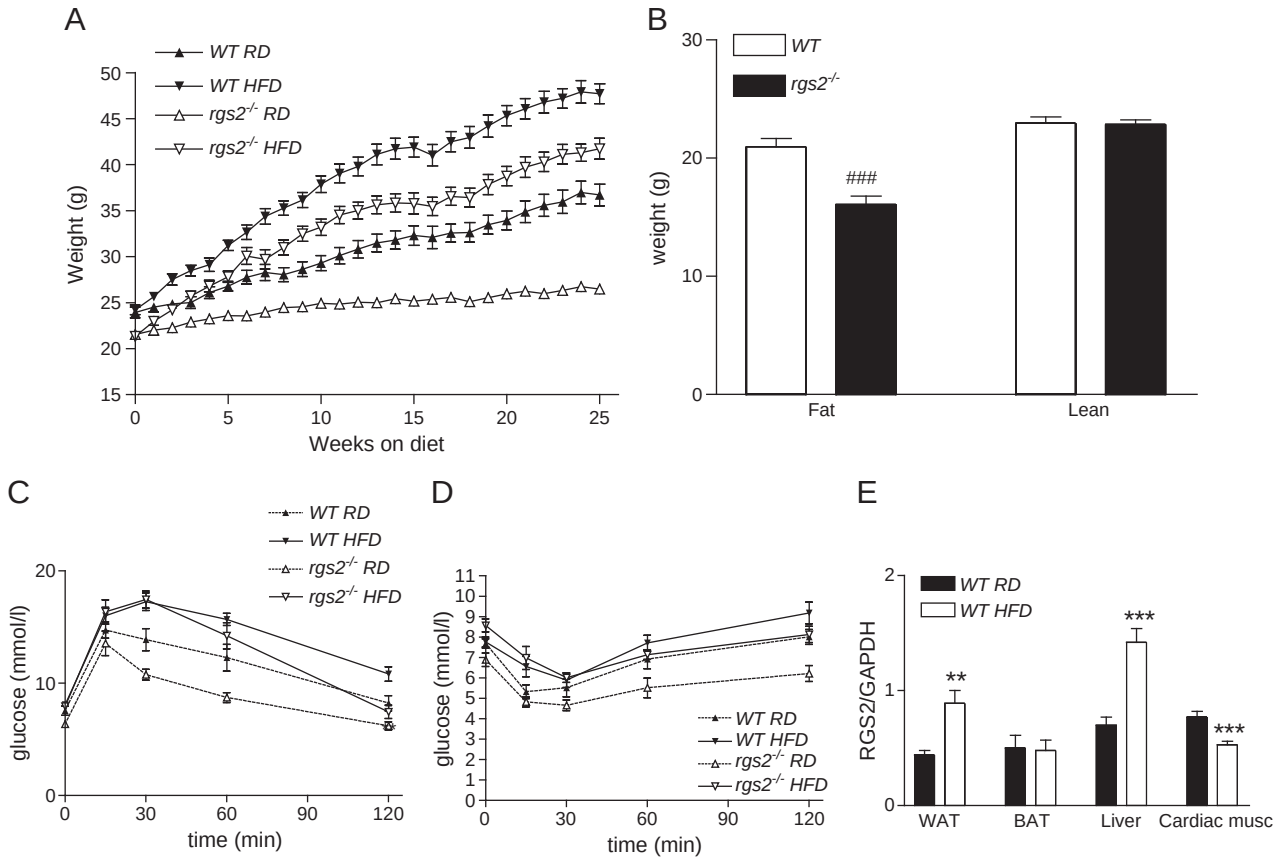

Fig. 9. High fat diet. (A) WT and *rgs2*$^{-/-}$ male mice were fed a high fat diet (HFD, 42% calories from fat) or regular diet (RD, 17% calories from fat) for 25 weeks from 9 weeks of age. (B) Fat and lean mass of male mice after 25 weeks on HFD determined using echoMRI scanning. ###$p<0.0001$ *t*-test comparing WT and *rgs2*$^{-/-}$. (C) Glucose tolerance test: male mice were fasted for 6 h before i.p. injection of glucose (1 mg/kg). (D) Insulin sensitivity test: male mice were fasted for 2 h before s.c. injection of insulin (0.4 IU/kg). (E) real-time PCR determination of RGS2 expression in white adipose tissue (WAT), brown adipose tissue (BAT), liver and cardiac muscle from high fat or regular diet fed animals. **$p<0.01$, ***$p<0.0001$ *t*-test comparing RD and HFD. Data represent means ± S.E.M., n ≥ 8.

and/or reduced efficiency of energy utilization. Previous reports on *rgs2*$^{-/-}$ mice found no difference in physical activity between the wild-type and knockout mice [26,30] and so increased energy expenditure might be expected at the resting metabolic level. Surprisingly, data from O_2 consumption experiments suggest that basal metabolism is modestly reduced in the *rgs2*$^{-/-}$ mice. It may be noted that although some other studies of lean mouse models have found no difference in diurnal O_2 consumption, nocturnal metabolism was found to be significantly increased [42,43] and so it is possible that nocturnal metabolism may be altered in the *rgs2*$^{-/-}$ mice. Still, no apparent effect on circadian activity, exploratory behavior, or motor coordination was observed in a previous study on *rgs2*$^{-/-}$ mice [26], although the present results suggest that a re-evaluation of these may be in order. A further technical limitation is that wild-type and knockout animals may have responded differently to the collection chamber environment, as anxiety levels may differ between the two strains [26,30].

In summary this paper presents the novel finding that RGS2 is important in body fat regulation and insulin sensitivity; the exact mechanism by which this occurs is currently not understood and further experiments should elucidate this. In particular, assessments of animal movement and O_2 consumption over longer periods and particularly at night might reveal additional between-strains differences, and further analyses of gene expression may reveal compensatory changes in relevant proteins [54]. Another possible contributing factor to the lean phenotype is that *rgs2*$^{-/-}$ mice may expend an increased amount of energy due to a greater rate of protein turnover (due to the lack of an RGS2 inhibitory effect on the initiation of mRNA translation), and indeed we have shown that hepatocytes isolated from these animals exhibit an increased rate of de novo protein synthesis [20].

Obesity is a growing problem in the Western world with as many as 2/3 of the US population being obese or overweight (National Centre for Health Statistics) and the direct medical costs of obesity estimated to be as high as $92 billion in 2002 [56]. The importance of the current study with regard to the problem of human obesity is as yet unknown, however, a recent study from the European Project on Genes in Hypertension found that a common substitution mutation in the promoter region of human RGS2 which leads to increased expression of the protein, increases susceptibility to the metabolic syndrome [57] suggesting that increased RGS2 may be involved in obesity and metabolic disease in humans and may be an important target for the future development of anti-obesity strategies.

Acknowledgements

We acknowledge Jari Tuomi for the initial observation and thank Alex Gerson for technical assistance with respirometry and fat/lean mass scans, Dr. Ian Welch for assistance with mouse pathology, Dr. Jim Staples for use of temperature controlled animal holding rooms, Shannon Mifflin for technical assistance, Dr. Haibo Wang for technical advice on thermogenesis experiments, Erin Mulvihill for advice on insulin and glucose tolerance tests, Richard Harris for microscopy assistance, Ting Zhang for technical advice on adipocyte isolation and differentiation experiments and Dr. Sheila Collins, Dr Wenhong Cao, Dr. Subrata Chakrabarti, Dr. Robert Gros and Dr. Murray Huff for

helpful discussions. Parts of this study were presented by CN in a short abstract talk at the Third RGS Colloquium, San Diego, April 2008.

This work was supported by the Canadian Institutes for Health Research (CIHR) and the Heart and Stroke Foundation of Ontario (HSFO) (PC), CN was supported by a CIHR postdoctoral fellowship. PC holds a Career Investigator Award from HSFO.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.cellsig.2011.03.020.

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