

Resistance to high fat diet-induced obesity but exacerbated insulin resistance in mice overexpressing Pref-1: a new model of partial lipodystrophy

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Submitted 12 December 2007 and accepted 17 September 2008.

Additional information for this article can be found in an online appendix at
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ABSTRACT

Objective. White adipose tissue is a critical regulator of whole body glucose metabolism. Pref-1 is a secreted protein that inhibits adipocyte differentiation, both in vitro and in vivo. In this study, we have investigated the effects of Pref-1 overexpression on whole body glucose homeostasis and its contribution to the development of insulin resistance.

Research Design and Methods. To gain insights into the role of Pref-1 on the onset of insulin resistance and type 2 diabetes, we measured body composition and whole body insulin-stimulated glucose metabolism during a hyperinsulinemic-euglycemic clamp in Pref-1 transgenic and wild type control mice fed a high fat diet.

Results. Mice overexpressing Pref-1 were resistant to high fat diet-induced obesity as reflected by a marked reduction in adipose tissue mass. However, Pref-1 overexpressing mice were severely insulin resistant, mainly due to a reduction in insulin-stimulated glucose uptake in skeletal muscle and adipose tissue. The aggravated insulin resistance was associated with impaired insulin signaling and increased diacylglycerol content in skeletal muscle.

Conclusions. Mice overexpressing Pref-1 are insulin resistant despite being protected from diet-induced obesity and may provide a new rodent model for the study of lipodystrophic disorders.

The prevalence of type 2 diabetes is rapidly increasing worldwide and it has been predicted that more than 366 million people will be affected by the year of 2030 (1). Although the primary cause of type 2 diabetes is not well defined, it is believed that insulin resistance plays a key role in the development of the disease (2). The mechanisms that trigger insulin resistance remain poorly understood but evidence points out to alterations in adipose tissue function and a concomitant ectopic lipid accumulation in muscle and liver as one of the principal underlying causes.

White adipose tissue (WAT) serves as the main energy storage depot of the organism. Energy excess stored in the form of triglycerides is released in periods of scarcity to supply the energy needs of other tissues. However, adipose tissue also functions as an active endocrine organ by secreting a variety of biologically active molecules such as leptin, adiponectin, ADSF/resistin, TNF α , IL-6 and PAI (reviewed in (3; 4)). These adipokines regulate multiple and crucial aspects of the organism's physiology, including appetite, energy metabolism, immune function and reproduction. Hence, adipose tissue is now recognized as a key player in the regulation of energy balance and glucose homeostasis (5). Indeed, alterations in the capacity of adipose tissue to store triglycerides or to synthesize/secrete adipokines have been linked to the appearance of metabolic disease, particularly insulin resistance and type 2 diabetes (6).

Lipodystrophies, a family of congenital or acquired disorders characterized by total or partial loss of adipose mass (reviewed in (7)), represent one of the best paradigms of adipose tissue dysfunction. Patients affected by lipodystrophy exhibit metabolic complications that include insulin resistance, hyperlipidemia or diabetes. The pathogenic basis of most of the lipodystrophies remain

still unknown, and only mutations in a few genes, such as lamin A/ C (8), AGPAT2 (9), seipin (10) or PPAR γ (11), have been identified as cause of congenital lipodystrophies. These genes regulate different aspects of adipose cell biology, particularly metabolism, differentiation and survival of adipocytes, underscoring that terminal maturation and proper functionality of adipocytes are essential requirements for appropriate whole body lipid and glucose homeostasis.

The mechanisms that control adipocyte differentiation are complex. However, several critical transcriptional regulators and extracellular signals that regulate adipocyte differentiation have been identified (reviewed in (12; 13)). Among them, our laboratory identified Preadipocyte factor-1 (Pref-1) (14) as an inhibitor of adipocyte differentiation, both *in vitro* and *in vivo* (reviewed in (4)). Pref-1 is synthesized as a transmembrane protein which EGF repeat-containing ectodomain is cleaved by tumor necrosis factor- α converting enzyme (TACE) to release a biologically active 50 kDa soluble form (15). Soluble Pref-1 functions in a paracrine/endocrine manner to prevent preadipocyte differentiation through MEK/ERK activation (16; 17). Mouse models of loss or gain of function have unequivocally demonstrated the important role of Pref-1 in adipogenesis. Mice lacking Pref-1 show growth retardation and skeletal abnormalities as well as increased adiposity when fed a high fat diet (18), supporting the role of Pref-1 on the regulation of adipocyte differentiation. Accordingly, young adult mice that overexpress soluble Pref-1 exhibited a marked reduction in WAT mass as a result of impaired adipocyte differentiation (19). Interestingly, these mice also showed skeletal malformations, impaired whole body insulin sensitivity and reduced glucose tolerance. These reports suggest that alterations in

circulating Pref-1 levels can impact whole body glucose homeostasis. However, the effect of Pref-1 on glucose homeostasis, particularly in individual tissues, or the underlying mechanisms of such metabolic alterations have not been explored.

Here, we examined the effects of Pref-1 overexpression on insulin action and glucose and lipid metabolism in mice that have been chronically fed a high fat diet. We found that mice overexpressing Pref-1 were insulin resistant despite a decrease in fat mass. Therefore, Pref-1 transgenic mice may provide a new rodent model of partial lipodystrophy.

RESEARCH DESIGN AND METHODS

Animals. Generation of transgenic mice (Tg) overexpressing the Pref-1/hFc fusion protein driven by the adipose-specific aP2 promoter has been previously described (19). Wild type (Wt) and transgenic littermates were fed a high-fat diet (45 % kcal fat, 35 % kcal carbohydrate, 20 % kcal protein) (Research Diets, New Brunswick, NJ) *ad libitum* for a period of 17 weeks after weaning. Food intake was measured every two days over a 10-day period in 15-week old male mice. All procedures involving animals were conducted in accordance with the institutional animal use and care guidelines of the University California-Berkeley and the Yale University School of Medicine.

Body composition. Fat and lean body mass was assessed by ^1H magnetic resonance spectroscopy (Bruker BioSpin, Billerica, MA). The mass of major adipose depots (gonadal, inguinal, and retroperitoneal depots) was directly measured by weighing the tissues after dissection.

Adipose tissue histology. Inguinal WAT from 20-week old Pref-1 Tg mice and Wt littermates was isolated and fixed overnight in Bouin's solution. After dehydration, the tissue was embedded in paraffin for subsequent

sectioning. 8 μm sections were stained with hematoxylin-eosin.

Serum metabolites. Blood from Pref-1 Tg mice and Wt littermates random-fed or fasted for 5 h was collected from tail and centrifuged at 3000 rpm for 5 min to obtain serum. Triglyceride levels were measured with the Infinity triglyceride reagent (Sigma-Aldrich, St. Louis, MO). Free fatty acids (FFA) were determined with the NEFA-C kit (Wako, Richmond, VA). Circulating insulin and leptin levels were measured using a rat insulin and mouse leptin ELISA kit (Crystal Chem, Downers Grove, IL), respectively. For adiponectin, a rat/mouse ELISA kit (B-Bridge, Sunnyvale, CA) was used. Blood glucose was measured with a glucometer (AccuCheck, Roche, Indianapolis, IN).

Glucose tolerance test. Glucose tolerance test was performed on 12 h fasted mice. Blood glucose was measured at 0, 30, 60, 90 and 120 min after an intraperitoneal injection of glucose (1g/kg body weight).

Hyperinsulinemic-euglycemic clamp study. Seven days prior to the hyperinsulinemic-euglycemic clamp studies, indwelling catheters were placed into the right internal jugular vein. After an overnight fast, [^3H]-glucose (HPLC purified; Perkin Elmer, Boston, MA) was infused at a rate of 0.05 $\mu\text{Ci}/\text{min}$ for 2 hours to assess the basal glucose turnover. Following the basal period, hyperinsulinemic-euglycemic clamp was conducted for 120 min with a primed/continuous infusion of human insulin (105 pmol/kg prime, 15 pmol/kg/min infusion) (Novo Nordisk, Princeton, NJ). Blood samples (10 μl) were collected at 10-20 min intervals for the immediate measurement of plasma glucose, and 20% dextrose was infused at variable rates to maintain plasma glucose at basal concentrations (~ 6.7 mM). To estimate insulin-stimulated whole body glucose fluxes, [^3H]-glucose was infused at a rate of 0.1 $\mu\text{Ci}/\text{min}$ throughout the clamps and 2-deoxy-D-[1- ^{14}C]glucose (Perkin Elmer,

Boston, MA) was injected as a bolus 75th minutes into the clamp to estimate the rate of insulin-stimulated tissue glucose uptake as previously described (20). Blood samples (10 μ l) for the measurement of plasma ³H and ¹⁴C activities were taken at the end of the basal period and during the last 45 min of the clamp. At the end of the clamp, mice were euthanized and tissues removed for biochemical measurements.

Analysis of Insulin signaling. After an overnight fast, Wt and Pref-1 Tg mice were intraperitoneally injected with saline or 0.85 U/Kg of insulin and euthanized 10 min after. Lysates from WAT, liver and skeletal muscle were obtained in homogenization buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM Tris pH 7.4) containing a protease and phosphatase inhibitor cocktail (Sigma, St. Louis, MO). Thirty to sixty μ g of protein were subjected to SDS-PAGE and immunoblotted with specific antibodies for total Akt or Akt phosphorylated at residue Ser473 (Cell Signaling, Danvers, MA). For IRS phosphorylation, lysates (1mg protein) were first immunoprecipitated with specific antibodies against IRS-1 or IRS-2 (Millipore, Billerica, MA). Immunoprecipitates were then subjected to SDS-PAGE and blotted with 4G10 antibody (Millipore) that detects phosphorylated tyrosine residues or antibodies that detect total IRS-1 or IRS-2.

Akt activity. Tissue lysates (500 μ g protein) were subjected to immunoprecipitation for 4h at 4°C with 5 μ l of a polyclonal Akt antibody (Upstate Biotechnology), coupled to protein A-sepharose beads. The immune complex was washed, and Akt activity was determined as described (21).

Lipid metabolites. Tissue triglycerides were extracted using the method of Bligh and Dyer (22) and content was measured using a DCL Triglyceride Reagent (Diagnostic Chemicals, Oxford, CT). Fatty Acyl-CoAs, diacylglycerols and ceramides extraction and

measurement by LC/MS/MS have been described previously (23).

Gene expression. RNA was isolated from skeletal muscle, WAT and liver and cDNAs were synthesized with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) and oligo dT. Gene expression was assessed by real-time quantitative PCR using specific primers and TaqMan probes for FATP-1, -2, -4, -5 and CD36 (Applied Biosystems). Quantification was performed by the $\Delta\Delta$ CT threshold cycle method and relative gene expression was normalized to GAPDH levels.

Statistical analysis. Results are expressed as mean \pm SEM. Statistical significance of differences between experimental groups was assessed using unpaired student's *t* test.

RESULTS

Pref-1 overexpressing mice are resistant to diet-induced obesity. We recently reported that overexpression of a Pref-1/hFc fusion protein in mice impaired adipocyte differentiation (19). Interestingly, these mice exhibited a mild degree of glucose intolerance and insulin resistance at young age (~10 weeks old). To further investigate the effects of Pref-1 overexpression on tissue-specific insulin sensitivity, we carried out comparative studies on Pref-1 Tg mice and Wt littermates fed a high fat diet for 17 weeks. High fat diets are known to induce obesity and to promote insulin resistance and diabetes in mice and humans, particularly if individuals are subject to such diets for a long period of time. At weaning, Pref-1 transgenic male mice weighed slightly less than Wt littermates (Wt=9.5 \pm 0.5 g, n=17 vs Tg=8.4 \pm 0.5 g, n=15; *P*=0.074) (Figure 1A). After 17 weeks of high fat diet feeding, Wt mice became evidently obese, exhibiting an average of more than 8 g in body weight above Pref-1 transgenic mice, which remained considerably leaner (Wt=43.1 \pm 1.1 g, n=17 vs Tg=34.8 \pm 1.3 g, n=15; *P*<0.01). Similarly, female transgenic mice were also resistant to diet-

induced obesity (Wt=34.2 ± 1.0 g, n=10 vs Tg=28.5 ± 1.5 g, n=10; $P<0.01$) (Figure 1B). The resistance to high fat diet-induced obesity occurred despite similar food intake (Wt=0.420 ± 0.04 Kcal/g/day, n=6 vs Tg=0.417 ± 0.03 Kcal/g/day, n=7).

Compared to Wt, Pref-1 Tg mice exhibited a significant reduction in the mass of the major WAT depots (Figure 2A), such as gonadal, inguinal, and renal fat. The interscapular brown adipose tissue was also significantly decreased. The reduction in adipose tissue mass was accountable for most of the decrease observed in body weight, since ^1H magnetic resonance spectroscopy revealed no differences in lean mass between Wt and Pref-1 Tg mice (Figure 2B). Fat depots of Pref-1 transgenic mice appeared normal by gross morphological examination, although adipocytes were significantly smaller than those of Wt (Figure 2C). In addition, we have previously shown that expression levels for adipocyte markers including PPAR γ , C/EBP α and FAS are lower in adipose tissue of Pref-1 transgenic mice, indicating impairment of adipocyte differentiation (19). The reduction in adipose tissue mass and adipocyte size in Pref-1 transgenic mice was accompanied by an increase in the circulating levels of free fatty acids and triglycerides, in both fasted and fed states (Table I), reflecting the fact that Pref-1 transgenic mice have reduced capacity to store triglycerides in adipose tissue.

Dysregulated glucose homeostasis in Pref-1 transgenic mice. The inability of adipocytes to properly store fat is frequently linked to alterations in glucose homeostasis and development of insulin resistance. Although fasting plasma glucose levels were similar in Pref-1 Tg and Wt mice (Table I), glucose tolerance test showed that transgenic mice have an impaired capacity to clear glucose from circulation, compared to Wt mice (Figure 3A). To investigate the mechanisms of glucose intolerance, a 2h-

hyperinsulinemic-euglycemic clamp assay was conducted on awake Wt and Pref-1 transgenic littermates. During the clamp, plasma insulin level was increased to ~330pmol and glucose levels were maintained at ~120 mg/dL by variable infusion of glucose. The glucose infusion rate necessary to maintain euglycemia in Pref-1 Tg mice was 50 % lower than in Wt, indicating that insulin-stimulated glucose uptake and metabolism was severely blunted in transgenic mice (Figure 3B). This could be attributed to a 40 % decrease in insulin-stimulated whole body glucose uptake in Pref-1 transgenic mice (Figure 4A). Because hepatic glucose production (HGP) was similar in Pref-1 Tg and Wt mice during the clamp as well as in basal condition, the inability of insulin to suppress HGP during the clamp in either Wt or Pref-1 Tg mice suggests that both experimental groups of mice developed severe but similar hepatic insulin resistance (Figure 4B). Supporting a similar HGP, no differences in the expression of key gluconeogenic genes *Pepck* and *G6pc3* between Pref-1 transgenic mice and wild type littermates were found (data not shown). On the other hand, consistent with the differences observed in whole body glucose uptake between Wt and Pref-1 Tg mice, insulin-stimulated glucose uptake was significantly reduced in skeletal muscle and WAT of Pref-1 transgenic mice compared to Wt (Figure 4C, D).

Impaired insulin signaling and increased lipid metabolites in skeletal muscle of Pref-1 transgenic mice. The action of insulin on glucose metabolism in peripheral tissues relies on the proper activation of the insulin signaling pathway and the correct elicitation of responses by molecular targets that eventually lead to glucose transport and metabolism. To investigate if the exacerbated insulin resistance present in Pref-1 transgenic mice is due to defects in insulin signaling, we analyzed the insulin-stimulated IRS and Akt

phosphorylation in a variety of insulin-sensitive tissues (Fig 5A and B). Insulin administration, although at a reduced degree, similarly increased phosphorylation of IRS-2 and Akt in liver of Wt and Tg mice, indicating comparable degree of hepatic insulin sensitivity. In WAT, insulin-stimulated phosphorylation of IRS-1 and IRS-2, as well as Akt, was approximately 50% lower in Pref-1 Tg mice compared to Wt littermates. More significantly, phosphorylation of IRS-1 and Akt upon injection of insulin was severely blunted by 80 % in skeletal muscle of Pref-1 Tg mice compared to Wt (Figure 5A, B). Consistent with these observations, a 40% reduction in Akt activity was observed in gastrocnemius muscle of Pref-1 Tg mice compared to Wt (Figure 5C). Similarly, Akt activity in WAT tended to be 40 % lower in Tg mice. On the other hand, there was no difference in liver Akt activity in Pref-1 Tg and Wt mice (Figure 5C). Together, these results demonstrate that in Pref-1 Tg mice insulin action in WAT and skeletal muscle, the latter being the major contributor to glucose utilization in the organism, is strongly impaired.

Evidence suggests that increased lipid accumulation in non-adipose tissues plays a major role in the development of insulin resistance associated with obesity and lipodystrophy. As shown in Table 1, circulating free fatty acid and triglyceride levels were higher in Pref-1 transgenic mice, presumably due to the significant reduction in lipid storage capacity of adipose tissue in these mice. To better understand how the metabolic alterations observed in mice overexpressing Pref-1 can inhibit insulin signaling and induce insulin resistance, we analyzed lipid metabolites content in liver and gastrocnemius muscle of Wt and Pref-1 transgenic mice. In liver, no significant difference in diacylglycerols (DAG) or fatty acyl-CoAs were found (Figure 6A), although triacylglycerols and ceramides content was

somewhat reduced (25 % and 17 %, respectively). In skeletal muscle, we did not observe any significant difference in triacylglycerols, fatty acyl-CoAs or ceramides content (Figure 6B) between Wt and Pref-1Tg mice. However, we detected an increase of nearly 60% in total DAG content in muscle of Pref-1 transgenic mice (Figure 6C). Elevated DAG level in skeletal muscle has previously been shown to cause insulin resistance after lipid infusion or fat feeding in rodents (24-26) as well as humans (27). This suggests that the increased levels of DAG observed in the skeletal muscle of Pref-1 transgenic mice could be a contributing factor for the aggravated insulin resistance associated with the lipodystrophy present in Pref-1 transgenic mice.

We next examined whether changes in the expression of proteins involved in flux of fatty acids into the cell, including FATPs and CD36, contribute to lipid accumulation in tissues of Pref-1 Tg mice. Interestingly, we found a 4-fold increase in CD36 expression in muscle, but not in liver, while no difference was observed in the expression of FATP family members known to be expressed in each of these tissues (Figure 7A and B). In addition, a substantial decrease in FATP1 and CD36 mRNA was found in WAT of Pref-1 Tg mice (Figure 7C), probably due to the impairment in adipocyte differentiation and lipid accumulation observed in Pref-1 Tg mice. These results suggest that higher CD36 expression in muscle of Pref-1 Tg mice, together with increased lipid availability, may contribute to the preferential lipid, namely DAG, accumulation observed in the skeletal muscle of Pref-1 Tg mice.

DISCUSSION

In this study, we show that high levels of circulating Pref-1 prevent the body weight gain and adipose tissue accumulation that are normally associated with high fat diets. However, similar to other models of

lipodystrophy, the resistance to diet-induced obesity exhibited by Pref-1 transgenic mice did not prevent the deleterious effects associated with feeding of a high fat diet, such as hyperlipidemia and insulin resistance. Indeed, compared to Wt littermates, Pref-1 transgenic mice showed an aggravated degree of whole body insulin resistance with higher circulating lipid levels. A generalized decrease in adipose tissue mass together with insulin resistance, are defining traits of lipodystrophy (28). In this sense, Pref-1 transgenic mice may represent a novel rodent model of partial lipodystrophy.

It is evident that chronic feeding of a high fat diet promoted development of glucose intolerance and insulin resistance in both Wt and Pref-1 transgenic animals. This is illustrated, for instance, by the very low overall glucose infusion rate (GINF) required to maintain euglycemia during hyperinsulinemic-euglycemic clamp in Wt and Pref-1 transgenic mice fed a high fat diet, compared to Wt fed a standard chow diet (10% kcal fat) (data not shown). Indeed, in mice fed a high fat diet GINF oscillated between 12.1 and 5.4 mg/kg/min in Wt and Pref-1 Tg mice, respectively (Figure 3B), whereas the GINF required for maintaining euglycemia in a cohort of Wt mice fed a normal chow diet for the same period was approximately 3-6 fold higher (data not shown). In this sense, the lack of effect of insulin in inhibiting hepatic glucose production in both Wt and Pref-1 Tg mice indicates the presence of severe hepatic insulin resistance in both groups. This is supported by a weak phosphorylation of hepatic IRS-2 and Akt upon insulin stimulation and similar Akt activity in liver of both groups, compared to those observed in other tissues. Also, the similar degree of activation of the insulin signaling pathway in liver of Wt and Tg mice, together with similar levels of gluconeogenic gene expression in fed Wt and Tg mice, suggest a comparable

degree of hepatic insulin resistance with a minor contribution of liver to the differential whole body insulin sensitivity exhibited by Pref-1 Tg mice. On the other hand, overexpression of Pref-1 clearly worsens insulin sensitivity in skeletal muscle, which is considered to be the primary tissue for insulin-mediated glucose disposal, as well as in adipose tissue, as demonstrated by a decrease in glucose uptake, decreased insulin-stimulated IRS and Akt phosphorylation, and decreased Akt activity. Thus, the differential degree of insulin resistance in these two insulin-sensitive tissues of Pref-1 transgenic mice could account for the observed impairment in whole body glucose tolerance and insulin sensitivity in Pref-1 transgenic mice compared to Wt mice when fed a high fat diet.

Alterations in adipocyte functions, such as the capacity to store lipids and to secrete adipokines, have been proven to have major repercussions in the ability of the organism to regulate substrate fluxes in tissues, and undoubtedly have impacts on the onset of insulin resistance. Ectopic accumulation of lipids in insulin sensitive tissues other than adipose tissue is considered a major cause of the development of insulin resistance (2). There is a strong correlation between intramyocellular triglyceride accumulation and insulin resistance (29; 30). However, recent studies have shown that lipid metabolism intermediates other than triglycerides, including diacylglycerols (26; 27; 31), fatty acyl-CoAs (24; 32) and ceramides (33; 34), could be directly responsible for the inhibition of insulin signaling in the insulin resistant state. In this regard, compared to Wt, Pref-1 transgenic mice show a significant accumulation of diacylglycerols, but not fatty acyl-CoAs, triacylglycerols or ceramides, in gastrocnemius muscle, identifying DAG as a potential candidate for the inhibition of insulin signaling in muscle of Pref-1 Tg mice.

The mechanisms by which mice overexpressing Pref-1 accumulate DAG preferentially in muscle but not in other insulin-sensitive tissues are still unclear, although the high expression of the fatty acid transporter CD36 in muscle could be a factor contributing to lipid accumulation and insulin resistance in this tissue (35; 36). Nevertheless, a causative relationship between increased DAG accumulation and the development of insulin resistance in muscle of Pref-1 Tg mice cannot be unequivocally established, and other possible factors may need to be taken in account. For instance, the inability of adipocytes from Pref-1 Tg mice to secrete adequate levels of insulin-sensitizing adipokines, such as leptin and adiponectin, could also contribute to the insulin resistance observed in Pref-1 lipodystrophic mice. In this regard, future experiments of leptin and/or adiponectin replacement are needed in order to elucidate the precise role that alteration in leptin/adiponectin secretion plays in the development of insulin resistance in Pref-1 Tg mice. In addition, we have observed an increase in the expression of inflammatory cytokines, such as IL-6 and IL-1 β but not TNF α in WAT of Pref-1 Tg mice, although such increase does not seem to be accompanied by macrophage infiltration (assessed by expression of F4/80) (Supplemental Figure 1). Hyperlipidemia is often associated with the activation of inflammatory pathways and the appearance of insulin resistance (reviewed in (37)). Therefore, besides ectopic lipid accumulation or decreased endocrine function, a contribution of inflammatory pathways to the insulin resistance of Pref-1 Tg mice cannot be excluded.

The dysregulated lipid metabolism and the resulting alterations in glucose homeostasis in Pref-1 transgenic mice are attributable to the effects that Pref-1 has on the adipose tissue development, which is the major target of Pref-1 action. Our previous studies have

unequivocally demonstrated the critical role of Pref-1 in repressing preadipocyte differentiation into adipocytes (14; 17; 38). *In vivo*, repression of adipocyte differentiation by Pref-1 is manifested by reduced expression of mature adipocyte markers in WAT (19), including C/EBP α , aFABP or SCD, and the consequent reduction in the ability to store triglycerides and to secrete adipokines such as leptin and adiponectin (Figure 2 and Table I). The reduction in fat mass associated with high circulating levels of Pref-1 is not constrained only to our transgenic model but naturally occurring mutations that affect the expression of Pref-1 result in a similar phenotype in other species. Indeed, in sheep, a mutation of the intergenic region of chromosome 18 located between genes encoding for Pref-1 -also known as *dlk1* (39)- and the non-coding gene *Gtl2* increases the expression of the Pref-1/*dlk1* gene. The mutation results in the callipyge phenotype that is characterized by pronounced muscle hypertrophy and reduction of fat mass (40; 41). Also, in pigs, a polymorphism in the Pref-1/*dlk1* gene resulting in increased Pref-1 expression causes a decrease in fat deposition as well as an increase in lean muscle mass (42). Although we have not observed muscle hypertrophy in Pref-1 transgenic mice, our studies clearly suggest that the decreased fat mass observed in these models could be due to the inhibitory effect of Pref-1 on adipocyte differentiation. Similar phenotype, although more severe, has been observed in different rodent models for total or partial lipodystrophy, including PPAR γ 2 -KO (43), conditional PPAR γ^{ldi} KO (44), FAT-ATTAC mouse (45), aP2-DTA mouse (46; 47), aP2-nSREBP-1c (48) and aP2 A-ZIP/F1 fatless (49). These rodent models underscore the role of adipose tissue as an integrator and critical regulator of energy and glucose homeostasis in the organism. Most of these genetically engineered mice are good models for the study of severe or total lipodystrophy but to

date, only PPAR γ 2 KO and aP2-nSREBP-1c constitute acceptable models for partial lipodystrophy. The analogy between Pref-1 transgenic mice and the rodent models that are totally or partially devoid of adipose tissue, allow us to propose Pref-1 transgenic mice as a new additional model for partial lipodystrophy.

In humans, a strong correlation between the severity of insulin resistance and the extent of loss of adipose tissue has also been observed. So far, few genes responsible for human lipodystrophies have been identified. These include BSCL2/Seipin and AGPAT2, which are associated with the development of generalized lipodystrophy, as well as Lamin A/C and PPAR γ , which have been found to cause partial lipodystrophy. Yet, no direct association has been established between the expression of dlk1, the human homolog of Pref-1, and the appearance of congenital lipodystrophies. Interestingly, Pref-1 expression is increased in adipose tissue of HIV-1 infected patients that suffer from highly active antiretroviral treatment (HAART)-associated lipodystrophy (50), a syndrome that has a high incidence (between 25-50%) and is characterized not only by wasting of subcutaneous fat but also by hyperlipidemia and insulin resistance (51).

Although the possible implication of Pref-1/dlk1 in human lipodystrophic syndromes is very suggestive, further studies will be necessary to firmly establish the function of Pref-1 on the development of adipose tissue disorders in humans.

In conclusion, our present study shows that high circulating levels of the soluble form of Pref-1 prevents high fat diet-induced obesity but exacerbates insulin resistance, which is associated with accumulation of DAG and decreased insulin sensitivity in skeletal muscle and WAT. Mice overexpressing Pref-1 exhibit all the characteristics of other lipodystrophic models, including reduced adipose tissue mass, dyslipidemia and insulin resistance, and therefore could be considered as a model for the study of partial lipodystrophies.

ACKNOWLEDGEMENTS

This research was supported by RO1 DK-50828 and DK-75682 to H.S.S.; R01 DK-40936, U24 DK-76169 and P30 DK-45735 to G.I.S. G. I. Shulman is an investigator of The Howard Hughes Medical Institute and the recipient of a Distinguished Clinical Investigator Award from the American Diabetes Association.

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Table I. Serum Parameters in Fed and 5h-Fasted Pref-1 Transgenic (Tg) and Wild Type (Wt) Littermates.

	Fed		Fasted	
	Wt	Pref-1 Tg	Wt	Pref-1 Tg
Glucose (mg/dL)	n.d.	n.d	202 ± 11	214 ± 7
FFA (mEq/L)	0.406 ± 0.03	0.560 ± 0.03**	0.710 ± 0.08	0.950 ± 0.08 (<i>P</i> =0.06)
Triglycerides (mg/dL)	54.9 ± 3.7	64.4 ± 7.7	73.5 ± 7	90.2 ± 8.9
Insulin (pg/ml)	5010 ± 1041	3721 ± 819	3734 ± 525	3107 ± 554
Adiponectin (µg/ml)	24.0 ± 2.7	10.5 ± 0.8**	27.0 ± 3.1	10.1 ± 1.0**
Leptin (ng/ml)	28.0 ± 2.4	5.8 ± 0.9**	n.d.	n.d.

, *P*<0.05; *, *P*<0.01, n=6-7.

Figure 1. Body weight of wild type (black squares) and Pref-1 transgenic (grey circles) mice fed a high fat diet for 17 weeks. A: Males (n=15-17/group). B: Females (n=10/group).

Villena et al Figure 1

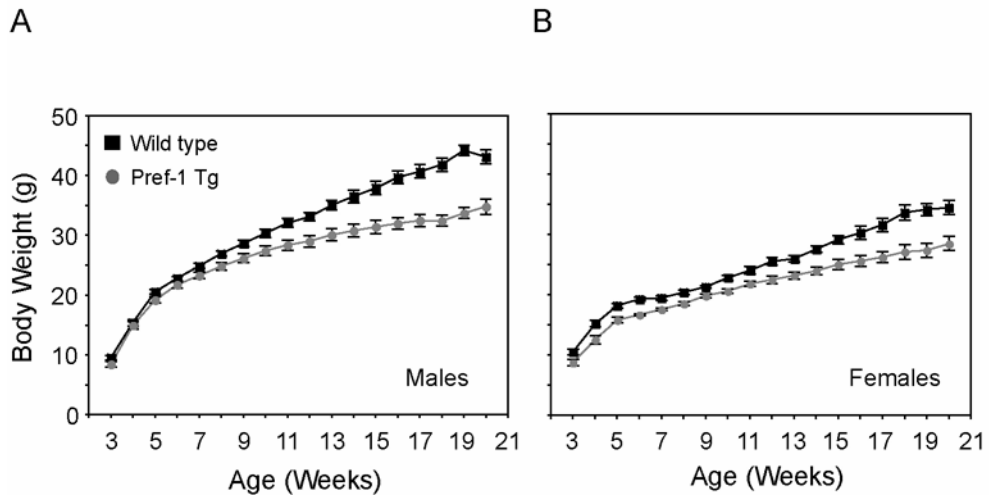


Figure 2. Protection from high fat diet-induced obesity in Pref-1 transgenic mice. A: Weight of major adipose tissue depots from Wt (black bars) and Pref-1 Tg (white bars) male (upper panel) and female (lower panel) mice. B: Fat mass and lean mass in Wt and Pref-1 Tg male mice measured by ^1H magnetic resonance spectroscopy (n=9/group). C: Paraffin-embedded sections stained with hematoxylin-eosin of inguinal white adipose tissue of Wt and Pref-1 Tg mice.

Villena et al Figure 2

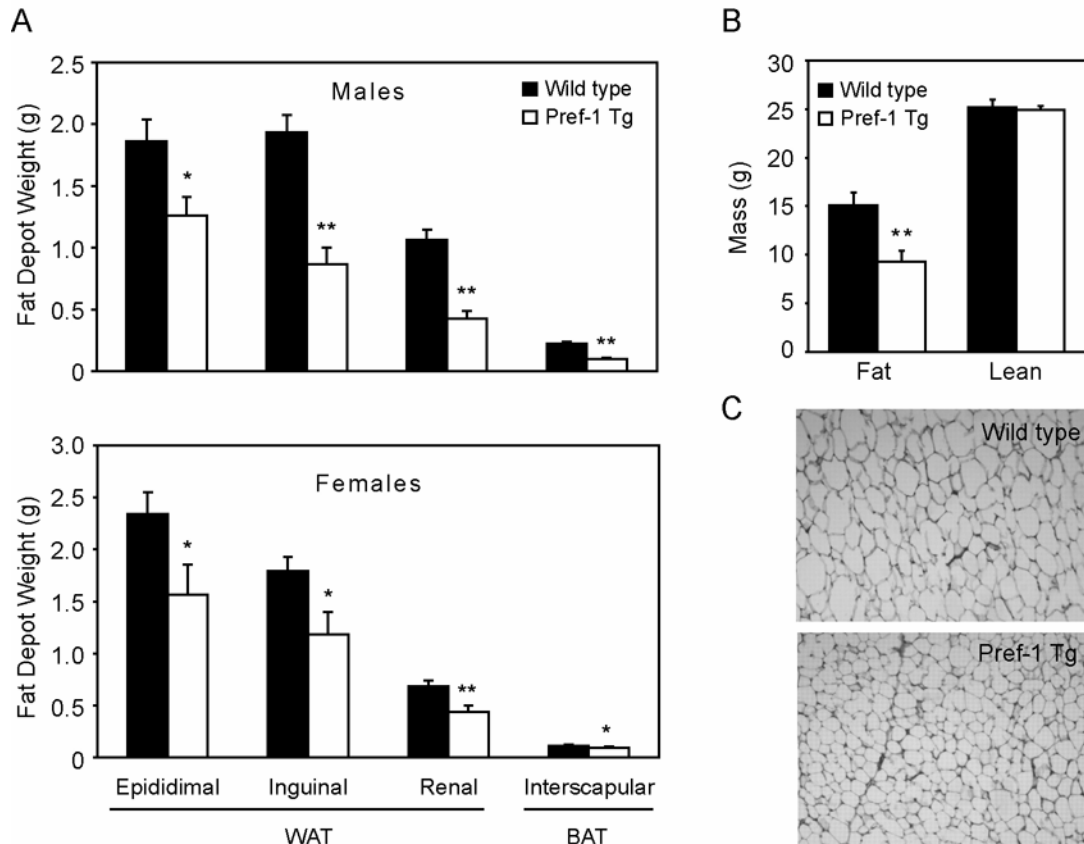


Figure 3. Glucose intolerance and insulin resistance in Pref-1 transgenic mice fed a high fat diet. A: Glucose Tolerance Test on Wt (black squares) and Pref-1 Tg (grey circles) mice, after 17 weeks on a high fat diet (n=6-7; *, $P<0.05$; **, $P<0.01$). B: Average glucose infusion rate (GINF) during the last 30 minutes of the hyperinsulinemic-euglycemic clamp assay in Wt (black bar) and Pref-1 Tg (white bar) mice (n=5-7/group. *, $P<0.05$).

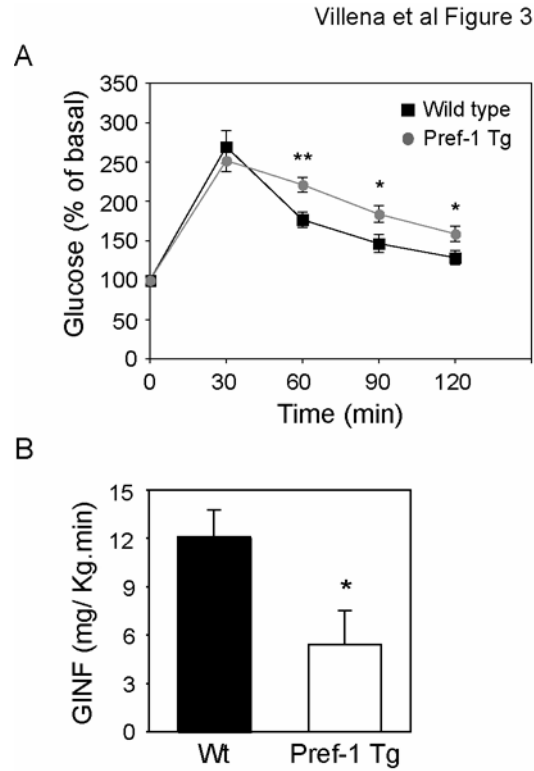


Figure 4. Glucose metabolism in Pref-1 transgenic (white bars) mice and wild type littermates (black bars) during hyperinsulinemic-euglycemic clamps. A: Insulin-stimulated whole body glucose uptake. B: Hepatic Glucose Production (HGP) prior and during the clamp. C: Glucose uptake by skeletal muscle (gastrocnemius). D: Glucose uptake by white adipose tissue.

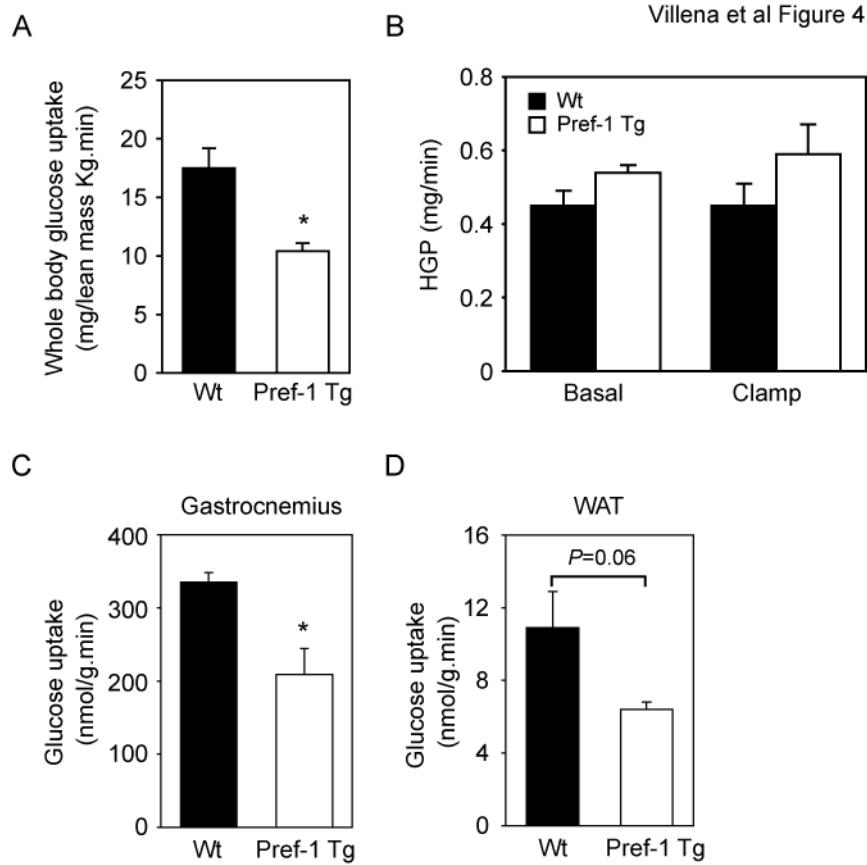


Figure 5. Insulin signaling pathway analysis. Mice were fasted overnight, then injected with saline or insulin (0.85U/Kg) and euthanized 10 min after injection. A: For analysis of insulin-induced phosphorylation of IRS, 1 mg of protein lysates from liver, WAT or skeletal muscle were first immunoprecipitated with anti-IRS-1 or anti-IRS-2 antibodies. Immunoprecipitates were then subjected to SDS-PAGE and blotted with anti IRS-1 in liver and WAT, IRS-2 in WAT and skeletal muscle to detect total levels of IRS-1 or IRS-2. Phosphorylation of IRS was detected in all tissues with an antibody specifically detecting phosphotyrosine residues. B: Total Akt and phosphorylated Akt were also detected by western blot in protein lysates of liver, WAT and skeletal muscle using specific antibodies against total Akt or phosphorylated Akt. C: Akt activity in skeletal muscle, WAT and liver. Tissue lysates (500 mg protein) were subjected to immunoprecipitation for 4h at 4°C with an Akt polyclonal antibody. Precipitated complexes were assayed for Akt activity as described previously (21). Results show mean \pm S.E.M of 4-6 animals per group. *, $P < 0.05$, #, $P < 0.09$.

Villena et al Figure 5

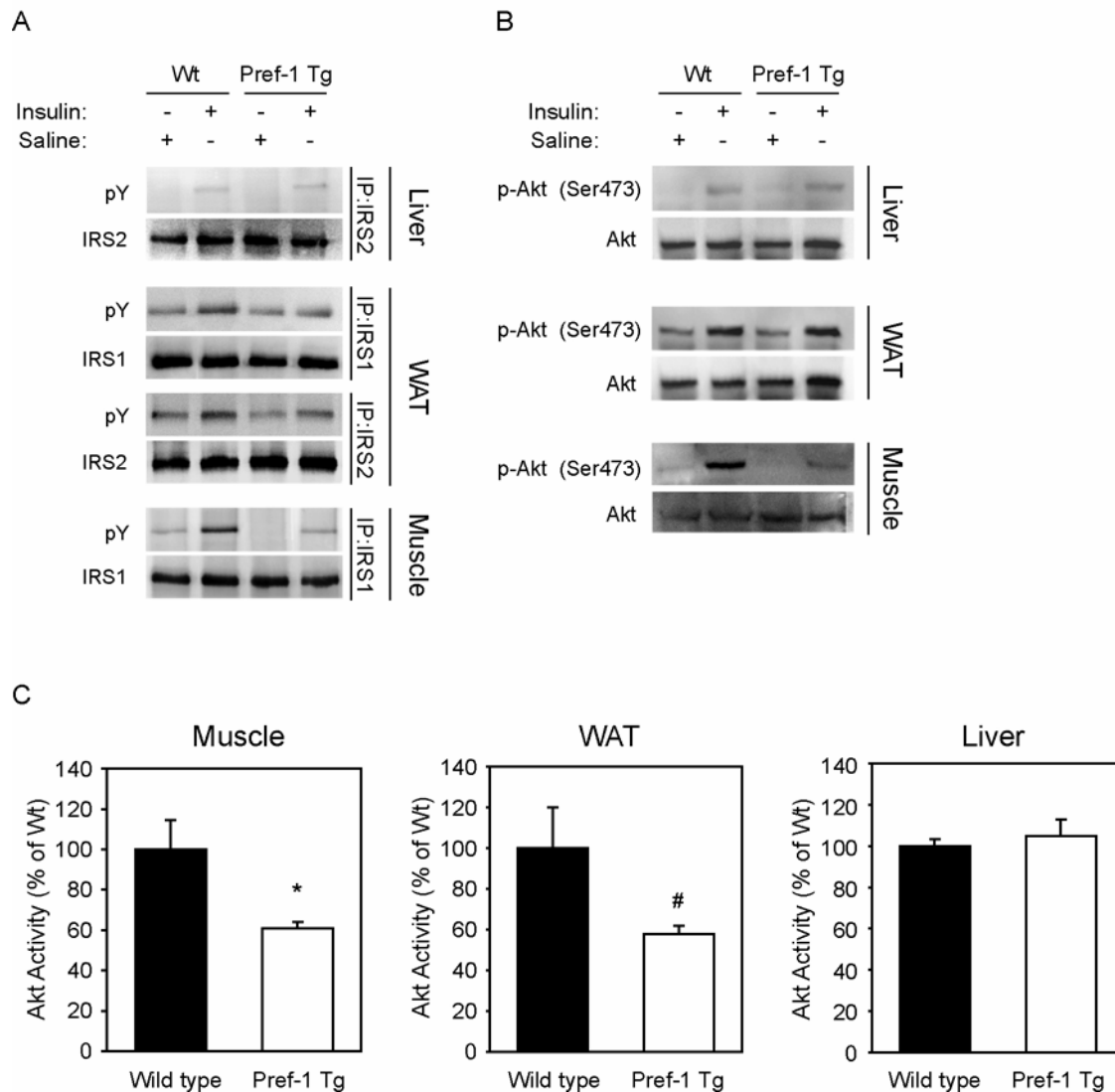


Figure 6. Lipid metabolites levels were measured in liver (A) and skeletal muscle (B) of Pref-1 transgenic (white bars) and wild type littermates (black bars) by LC/MS/MS. Results are expressed as mean \pm SEM of 7-8 animals/group. *, $P < 0.05$.

Villena et al Figure 6

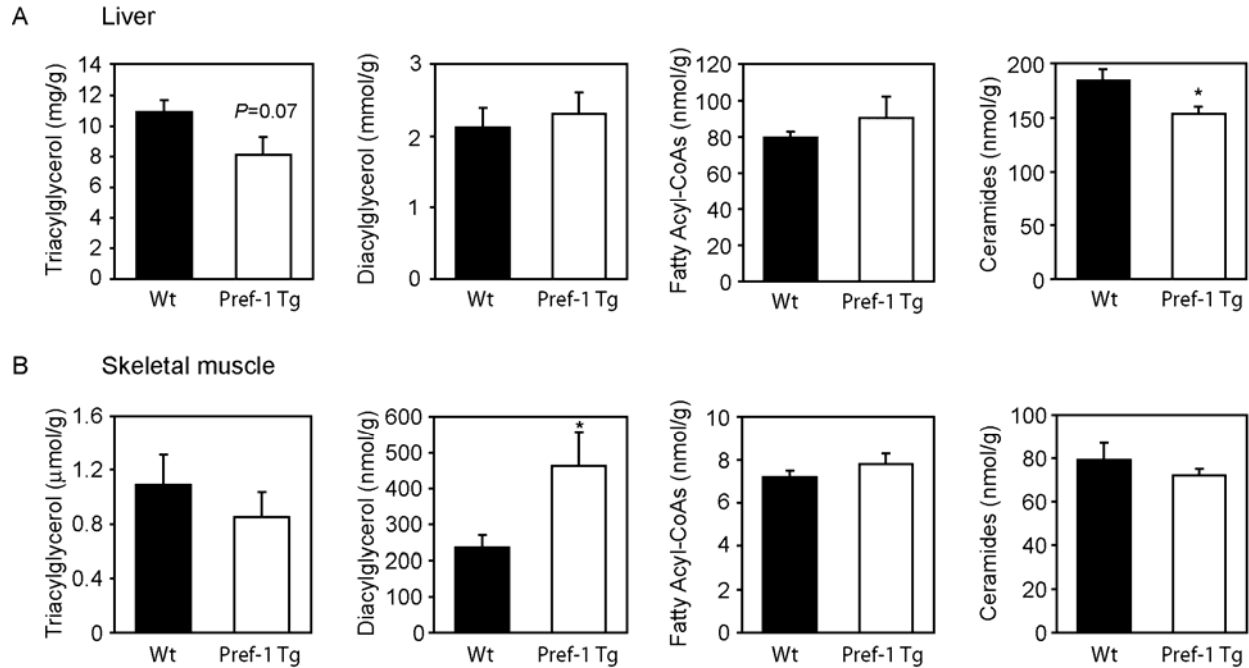


Figure 7. Expression levels of fatty acid transporters CD36 and FATPs in skeletal muscle (A), liver (B) and WAT (C) were assessed by real time quantitative PCR using specific primers and TaqMan probes. Fold-changes in comparison to the levels in Wt mice are shown and represent the mean \pm S.E.M. of 4-8 animals per group. *, $P < 0.05$.

