Agouti regulates adipocyte transcription factors

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Received 5 November 2000; accepted in final form 10 November 2000

Mynatt, Randall L., and Jacqueline M. Stephens. Agouti regulates adipocyte transcription factors. Am J Physiol Cell Physiol 280: C954–C961, 2001.—Agouti is a secreted paracrine factor that regulates pigmentation in hair follicle melanocytes. Several dominant mutations cause ectopic expression of agouti, resulting in a phenotype characterized by yellow fur, adult-onset obesity and diabetes, increased linear growth and skeletal mass, and increased susceptibility to tumors. Humans also produce agouti protein, but the highest levels of agouti in humans are found in adipose tissue. To mimic the human agouti expression pattern in mice, transgenic mice (aP2-agouti) that express agouti in adipose tissue were generated. The transgenic mice develop a mild form of obesity, and they are sensitized to the action of insulin. We correlated the levels of specific regulators of insulin signaling and adipocyte differentiation with these phenotypic changes in adipose tissue. Signal transducers and activators of transcription (STAT1, STAT3, and peroxisome proliferator-activated receptor (PPAR)-γ) protein levels were elevated in the transgenic mice. Treatment of mature 3T3-L1 adipocytes recapitulated these effects. These data demonstrate that agouti has potent effects on adipose tissue. We hypothesize that agouti increases adiposity and promotes insulin sensitivity by acting directly on adipocytes via PPAR-γ.

adipose tissue; signal transducers and activators of transcription; peroxisome proliferator-activated receptor; melanocortins

Several dominant agouti mutations in mice cause a phenotype characterized by yellow fur, mild hyperphagia, decreased thermogenesis, increased body-fat content, insulin resistance, impaired glucose tolerance, hyperglycemia, and increased susceptibility to tumors (48). The yellow obese syndrome seems to be caused by a paracrine factor because heterozygous parabiotic union between obese mutant mice and litter mates after 28 wk did not produce any changes in body weight or body composition compared with homozygous pairs (44). Cloning and sequence analysis revealed that the mouse agouti gene is located in the distal region of chromosome 2 and encodes for a small secreted protein (5). Agouti expression analysis of wild-type mice demonstrated that agouti is only expressed in the skin during the hair growth cycle (5). Examination of agouti mRNA from several dominant agouti mutations revealed structural changes in the promoter region of agouti that cause it to be expressed ubiquitously (5, 12, 24–26). However, because each of these dominant mutant alleles contains a major structural change in the agouti locus, it was unclear whether the ubiquitous expression of agouti per se causes the syndrome or whether there is an additional gene located in the vicinity of agouti that is altered. It was later demonstrated that agouti was responsible for the syndrome by ubiquitously expressing the wild-type agouti cDNA under control of β-actin and phosphoglycerate kinase-1 promoters in transgenic mice (21). These results demonstrated conclusively that the ectopic expression of agouti is solely responsible for the mutant traits in these animals.

The human homolog of the agouti gene is 85% identical to the mouse gene and encodes a protein of 132 amino acids with a consensus signal peptide (22). The major difference between mouse and human agouti is the expression pattern. Whereas mouse agouti is only transiently expressed in the hair follicle (5), human agouti is expressed in diverse tissues; primarily adipose tissue follow by the testis, heart, liver, kidney, ovary, and foreskin (22, 43).

The understanding of the mechanisms of agouti within the hair follicle has served as a paradigm for agouti-induced obesity. Within the hair follicle, α-melanocyte-stimulating hormone (α-MSH) binds to its receptor (MC1-R), which is coupled to the heterotrimeric guanine nucleotide-binding proteins that activate adenylate cyclase (9). The resulting increase in intracellular cAMP levels leads to the activation of the rate-limiting enzyme in melanogenesis, tyrosinase (16). Agouti decreases the overall rate of melanogenesis by antagonizing the binding of α-MSH to MC1-R and increases the incorporation of sulfhydryl compounds into dopaquinone to produce yellow pigment (17, 18).

Early experiments demonstrated that mouse adipocytes express high-affinity binding sites for melanocortin peptides (31). Boston and Cone (3) demonstrated that both MC2 and MC5 receptors are expressed in differentiated 3T3-L1 adipocytes and mouse adipose tissues but not in preadipocytes. Mountjoy and Wong (29) have shown expression of MC1, MC2, and MC5 receptor mRNA in differentiated 3T3F442A adipocytes

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using RT-PCR and in both white adipose tissue (MC1-R and MC2-R) and brown adipose tissue (MC2-R and MC5-R) in mice. The mRNA for all five melanocortin receptors was detected by RT-PCR in human subcutaneous adipose tissue (7). Expression of agouti protein and melanocortin receptors in human fat raises questions as to their normal functions in maintaining energy balance and whether a defect in the functioning of these receptors in fat might contribute to an obese, insulin-resistant, or diabetic phenotype.

ACTH, α-MSH, and β-lipotropin are potent lipolytic hormones (2). However, considerable species variability exists in the lipolytic response to melanocortins. The mouse adipocyte MC2 receptor exhibits properties similar to the ACTH receptor characterized in adrenocortical cells, coupling to activation of adenyl cyclase with an EC\text{50} of ~1 nM. Both ACTH and α-MSH bind to mouse adipocytes, but only ACTH elevates cAMP and stimulates lypolysis (2). Therefore, agouti antagonism of ACTH to adipocytes may lead to an inhibition of lipolysis and/or stimulation of lipogenesis. Data from Moustaid and colleagues (19) suggest that the agouti protein can increase lipogenesis in adipocytes. The mRNA levels of fatty acid synthase (FAS) and stearoyl-CoA desaturase (SCD), two key enzymes in de novo fatty acid synthesis and desaturation, respectively, were dramatically increased in obese (A\textsuperscript{770}) mice relative to lean (a/a) controls (19). Treatment of 3T3-L1 adipocytes with recombiant agouti protein increased FAS and SCD mRNA levels 1.5- and 4-fold, respectively (19). In addition, FAS activity and triglyceride content were threefold higher in agouti-treated 3T3-L1 cells relative to controls.

Experiments on the lipolytic effects of melanocortins in humans are in disagreement. Variables between the experiments included different fat depots, different fat cell isolation procedure and culture conditions, and different time courses. Experiments using adipocytes isolated from human intra-abdominal fat show no effects of α-MSH and ACTH on lipolysis in human adipose tissue (4). Xue et al. (47) investigated the role of agouti and ACTH in regulating lipolysis in primary cultures of adipocytes isolated from subcutaneous depots. Short-term (1 h) exposure to recombiant agouti protein had no effect on basal lipolysis, although longer term treatment (24 h) caused a 60% decrease in basal lipolysis. Short-term agouti treatment inhibited ACTH-induced lipolysis. This effect, combined with agouti-induced lipogenesis, may represent a coordinate control of adipocyte lipid metabolism.

The 3T3-L1 adipocytes are comparable to native adipocytes as they have the ability to accumulate lipid, respond to insulin and secrete leptin. The major transcription factors involved in adipocyte gene regulation include peroxisome proliferator-activated receptor (PPAR)\textsubscript{γ}, proteins belonging to the CCAAT/enhancer-binding protein (CEBP) family, and adipocyte determination and differentiation dependent factor 1, also known as sterol regulatory element binding protein (reviewed in Refs. 27 and 33). Recent studies have also suggested that the signal transducers and activators of transcription (STAT) family of transcription factors may also be important in fat cells. A STAT family member shows a distinct pattern of activation by cytokines and, upon nuclear translocation, can regulate the transcription of particular genes in cell- or tissue-specific manners (10). In fat cells, the expression of STAT1, STAT5A, and STAT5B is highly induced during differentiation and correlates with lipid accumulation (38, 40). The regulation of STAT expression has also been investigated in NIH/3T3 cells ectopically overexpressing CEBP-β and CEBP-δ, a condition that results in adipogenesis (45). In these studies, the expression of STAT1, STAT5A, and STAT5B was induced in a PPAR-γ ligand-dependent fashion during adipogenesis (39). STAT3 and STAT6 are also expressed in adipocytes, but the expression of these proteins does not change during differentiation. However, the tyrosine phosphorylation of STAT3 occurs after the induction of differentiation, and a study (11) with antisense STAT3 suggest that this protein may be important in adipogenesis. Although the functions of STATs in fat cells have not been identified, numerous studies suggest that these transcription factors are important regulators of adipocyte gene expression.

We (30) reported that transgenic mice that have the aP2-promoter driving agouti expression are sensitized to insulin. In this paper, we further describe the phenotype of the aP2-agouti transgenic mice and identified a potential mechanism of increased insulin sensitivity and obesity. These studies clearly indicate that agouti treatment of adipocytes results in the increased expression of three transcription factors. The most prominent effect of agouti on adipocyte transcription factors is the substantial increase in PPAR-γ expression in the fat pads of the aP2-agouti mice and in culture adipocytes that have been exposed to recombiant agouti protein. In addition, we observed an increase in both STAT1 and STAT3 in these conditions. Although the function of STATs in adipocytes has not been identified, it is known that these transcription factors can be activated in adipocytes and play a role in adipocyte differentiation (11, 37, 38). We hypothesize that the agouti-induced regulation of STAT1, STAT3, and PPAR-γ expression results in the regulation of various genes associated with the adipocyte phenotype.

**EXPERIMENTAL PROCEDURES**

**Mice.** Transgenic mice were generated at Oak Ridge National Laboratory (ORNL) as previously described (30). The aP212 and aP273 lines were rederived at Charles River Laboratory and maintained on the FVB/N background at Pennington Biomedical Research Center (PBRC; Baton Rouge, LA). All mice were fed a diet containing 11% fat by weight (Mouse Diet 5015, Purina Mills) and weaned at 21–25 days of age. Food and water were provided ad libitum. All data are from mice that are hemizygous for the transgene or their nontransgenic littermates. There were four litters examined per time point at 4, 6, and 10 wk, and two litters were examined in the 8-wk-old group. Transgenics were compared with littermates in every case but one at 4 wk, in which there were two transgenic males and no wild-type males in the litter. Mice were euthanized by cervical dislocation, and...
adipose tissue was quickly removed, weighed, and frozen in liquid nitrogen for future analysis. PCR genotyping and Northern blot hybridization analyses were performed as previously described (30).

Materials. Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Life Technologies (Grand Island, NY). Bovine and fetal bovine serum (FBS) were obtained from Sigma (St. Louis, MO) and Life Technologies, respectively. Murine agouti was a gift from Derril Willard of GlaxoWellcome Pharmaceuticals (Research Triangle Park, NC). The STAT antibodies were monoclonal IgGs purchased from Transduction Laboratories (Affiniti Research Products) or polyclonal IgGs from Santa Cruz Biotechnology (Santa Cruz, CA). PPAR-γ antibody was a mouse monoclonal from Santa Cruz.

Cell culture. Murine 3T3-L1 preadipocytes were plated and grown to 2 days postconfluence in DMEM with 10% bovine serum. Medium was changed every 48 h. Cells were induced to differentiate by changing the medium to DMEM containing 10% FBS, 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 1.7 μM insulin. After 48 h, this medium was replaced with DMEM supplemented with 10% FBS, and cells were maintained in this medium until utilized for experimentation.

Preparation of adipose tissue and whole cell extracts. Fat pads were homogenized in a nondenaturing buffer containing 150 mM NaCl, 10 mM Tris (pH 7.4), 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 0.5% Nonidet P-40, 1 μM phenylmethylsulfonyl fluoride, 1 μM pepstatin, 50 trypsin inhibitory milliunits of aprotinin, 10 μM leupeptin, and 2 mM sodium vanadate. Homogenates were centrifuged for 10 min at 10,000 rpm to remove any debris and insoluble material. Monolayers of fully differentiated 3T3-L1 adipocytes were rinsed with phosphate-buffered saline (PBS) and then harvested in the above nondenaturing buffer. Samples were extracted for 30 min on ice and centrifuged at 15,000 rpm at 4°C for 15 min. Supernatants containing whole cell and fat pad extracts were analyzed for protein content using a bicinchoninic acid kit from Pierce (Rockford, IL) according to the manufacturer’s instructions.

Gel electrophoresis and immunoblotting. Proteins were separated in 5, 7.5, or 12% polyacrylamide gels containing sodium dodecyl sulfate (SDS) according to Laemmli (23) and transferred to nitrocellulose in 25 mM Tris, 192 mM glycine, and 20% methanol. After the transfer, the membrane was blocked in 4% milk for 1 h at room temperature. Results were visualized with horseradish peroxidase-conjugated secondary antibodies from Sigma and enhanced chemiluminescence from Pierce.

RESULTS

In our original study (30) performed at ORNL, we did not observe any significant differences in body weight between transgenic mice that expressed agouti in adipose tissue (Tg/+), and their littermates (+/+), but the transgenic mice gained more weight if they were given insulin injections. The ORNL mice harbored a number of pathogens and parasites. Therefore, two transgenic lines were rederived at Charles River Laboratories and brought to the PBRC to establish new lines. Agouti expression in adipose tissue was confirmed in the rederived mice (Fig. 3A). Both nontransgenic and transgenic mice at PBRC were significantly heavier than their ORNL counterparts (Fig. 1). Additionally, the transgenic mice at PBRC have a statistically significant increase in body weight over the nontransgenic littermates (Fig. 1 (12 wk) and Fig. 2). The most probable explanation for the discrepancies in body weight reported from mice at ORNL and the mice at PBRC is the healthier status of the mice at PBRC. Examination of records from the ORNL mice revealed that many of the litters suffered from diarrhea and were given antibiotics in the drinking water. This immunological challenge during development caused a very large varia-

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**Fig. 1.** Comparison of body weights between mice at Oak Ridge National Laboratory (ORNL) and Pennington Biomedical Research Center (PBRC). Body weights are from mice housed at ORNL and mice that were rederived from the ORNL mice and established at PBRC. All mice were maintained on the FVB/N background, fed a diet containing 11% fat by weight, and weaned at 21–25 days of age. Data are from mice that are hemizygous for the transgene or their nontransgenic littermates. There were between 12 and 28 mice per data point. Data are presented as means ± SE. *Significantly different (P ≤ 0.01) from ORNL mice; **significantly different (P ≤ 0.01) from nontransgenic and ORNL mice.
tion in body weight between mice and between litters that reduced ability to detect a statistically significant change in body weight.

We choose the aP212 line to closely examine the cause for the increased body weight of the transgenic mice. Figure 2 compares both body weight and individual fat depot weights between transgenic mice and wild-type littersmates from 4 to 10 wk of age. The transgenic mice are heavier than littersmates at all times but become statistically different by 8–10 wk. The increased body weight correlates with an increased mass of all fat depots that becomes significant between 8 and 10 wk. Combined fat depot weight is increased 30–50% at 10 wk in individual transgenic mice compared with littermates. DNA content per gram of adipose tissue was reduced in the transgenic mice by 10 wk, and histological examination of fat depots revealed fat cell hypertrophy (data not shown).

Total RNA was extracted from the combined fat depots from 10-wk-old mice, and leptin mRNA levels were compared by Northern blot analysis (Fig. 3). In accordance with increased fat mass and fat cell size, leptin mRNA levels were substantially higher in the transgenic mice. These data demonstrate that the aP2-agouti transgene causes increased fat mass, resulting in mild obesity and increased leptin synthesis.

Members of both the STAT and PPAR family of transcription factors are regulated during adipogenesis and in conditions of altered insulin sensitivity (33). Therefore, we examined the levels of these transcription factors in retroperitoneal fat pads from transgenic mice and wild-type littersmates between 4 and 10 wk of age. In mice that were 4 wk of age, there were no detectable differences in the expression of STAT1, STAT5A, or PPAR-γ in wild-type and transgenic mice. However, by 6 wk of age, there was a discernible, but not statistically significant, increase in STAT1 expression in all of the transgenic mice compared with wild-type controls. PPAR-γ expression was elevated in three of five transgenic animals by 6 wk (data not shown).

We examined the data to see whether the differences were litter effects, and they were not. Because these mice are an inbred line, we assume they are genetically identical, and at this point it leaves only environmental effects to account for mouse-to-mouse variation. We have shown that this phenotype is “environmentally sensitive” by comparing ORNL versus PBRC mice. We can only speculate that factors such as birth order, nesting conditions, dominance during sucking and adulthood, and individual activity are affecting the phenotype. Interestingly, the increases in both STAT1 and PPAR-γ expression occurred before any effects in body weight and fat mass, suggesting that these transcription factors may be responsible for the accelerated accumulation of adipose tissue in the transgenic animals. By 10 wk of age, the expression of STAT1, STAT3, and PPAR-γ was significantly elevated in transgenic animals compared with nontransgenic littermates (Fig. 4A). Also, at 10 wk of age, there was a marked increase in body weight in the transgenic mice. To confirm these findings, a separate group of 10-wk-
old mice was examined. Both body weight \[32.66 \pm 0.54 \text{ g (Tg/+)} \] versus \[28.21 \pm 0.66 \text{ g (+/+)} \], \( P = 0.002 \) and retroperitoneal fat pad weight \[0.58 \pm 0.03 \text{ g (Tg/+)} \] versus \[0.33 \pm 0.04 \text{ g (+/+)} \], \( P = 0.003 \) were significantly greater in these transgenic mice compared with littermates. Western blots from both sets of mice were quantitated for statistical analysis (Fig. 4B). These results clearly demonstrate that STAT1, STAT3, and PPAR-\( \gamma \) expression was substantially elevated in transgenic mice compared with controls.

The \(-5.4\)-kb aP2 promoter exhibits low levels of ectopic expression in many tissues (30, 34), and the changes that we observed in adipose tissue may have been indirectly caused by ectopic agouti expression. To examine the direct effects of agouti on adipocytes, mature 3T3-L1 adipocytes were treated with recombinant murine agouti. Very similar to the adipose tissue from transgenic mice, STAT1, STAT3, and PPAR-\( \gamma \) were elevated in the agouti (50 nM)-treated 3T3-L1 adipocytes (Fig. 5). Yet there was no change in STAT5A levels after agouti treatment. Dose-response curves from 0.5 to 200 nM agouti demonstrated a near-maximum effect of agouti at 50 nM (data not shown), which is in agreement with inhibition of Nle\(^4\),D-Phe\(^7\)-\( \alpha \)-MSH binding to cells stably expressing mouse melanocortin receptors (20). The regulation of STAT1, STAT3, and PPAR-\( \gamma \) by the addition of recombinant agouti to cultured adipocytes unequivocally demonstrates that agouti directly influences adipocyte metabolism.

**DISCUSSION**

The agouti gene is expressed in adipose tissue in humans and has been shown to regulate lipid metab-
olism in cultured adipose cells in vitro (19, 47). Although the agouti gene is not normally expressed in adipose tissue in the mouse, we were able to induce the expression of high levels of agouti in white and brown adipose tissue by expressing the cloned mouse gene under the control of the aP2 promoter. The agouti expression in adipose tissue resulted in a phenotype quantitatively similar to middle-aged adiposity. The mice have a 10–15% increase in body weight caused by a 30–50% increase in fat mass. This would be equivalent to an extra 15 lb of body fat in a 150-lb person. These results indicate that the expression of agouti in adipose tissue has substantial metabolic effects and may be physiologically significant in humans.

To date, most models have focused on the central nervous system to explain the development of the yellow obesity syndrome in mice. Intracerebroventricular administration of α-MSH or a melanocortin analog, MTII, a potent agonist of the MC3 and MC4 receptors, suppresses feeding behavior in rodents, whereas injection of SHU9119, an antagonist of the same receptors, stimulates feeding (13). Inactivation of MC4-R by gene targeting (15) results in obese mice. Agouti-related peptide (Agrp) is a homolog of agouti found in the brain and adrenal cortex that also inhibits melanocortin signaling (32, 35). Overexpression of Agrp in mice results in an obese and diabetic phenotype very much like that seen in the A′ and MC4-R knockout mice (32, 35), suggesting that that ectopic expression of agouti in the hypothalamus mimics endogenous Agpr. The conclusion from these knockout and transgenic mice experiments was that the MC4-R/Agrp system is a major regulator of food intake and energy expenditure.

Recently, the MC3-R was inactivated resulting in decreased energy expenditure and increased fat mass, despite being hypophagic (6, 8). Perhaps the most striking observation was that the MC3 and MC4 pathways are not redundant. Mice lacking MC3-R are not heavier than their littermates until about 26 wk of age. In contrast, 26-wk-old mice lacking both MC3-R and MC4-R are significantly heavier than littermates lacking only MC4-R. The data suggest that in the absence of MC3-R, nutrients are preferentially partitioned into fat resulting in subtle body weight changes and almost a doubling of fat mass. The subtle change in body weight and increased fat mass phenotype in MC3-R knockout mice is very similar to our aP2-agouti transgenic mice. We predict that melanocortin signaling in adipose tissue is another nonredundant mechanism for body weight homeostasis and a cross between aP2-agouti and MC4-R/− mice will have a similar effect to the MC3-R knockout mice.

The most striking difference between transgenic and wild-type mice was the difference in PPAR-γ. Several studies (14, 27, 28, 36, 39, 42, 46) have shown that PPAR-γ is an essential transcription factor for differentiation and maturation of adipocytes. Additionally, ectopic expression of PPAR-γ in nonadipogenic fibroblast promotes lipid accumulation and characteristics of mature adipocytes (41). The elevated levels of PPAR-γ in the aP2-agouti mice are consistent with the increased adipocyte hypertrophy and increased insulin sensitivity. The elevated expression of STAT1 and STAT3 by agouti is interesting. Agouti does not appear to have any direct effect on STAT phosphorylation in adipocytes, but the expression of these two STATs has been shown to be highly regulated in fat cells (1). The expression of STAT1 is highly induced during adipocyte differentiation and can be controlled by thiazolidinedione treatment (38, 39). STAT3 may also be
imported in adipogenesis because STAT3 antisense oligonucleotides have been shown to inhibit fat cell differentiation (11). Although the functions of STATs in fat cells have not been identified, various studies suggest that these transcription factors are important regulators of adipocyte gene expression.

In summary, these data provide direct in vivo and in vitro evidence that agouti and/or melanocortin signaling may govern adipogenesis. These mice have significantly increased fat mass, accompanied by a substantial increase in three key adipocyte transcription factors that are also upregulated in agouti-treated 3T3-L1 adipocytes. The modest weight gain in these mice suggests that the normal hypothalamic pathways regulating food intake are intact and that the observed adiposity is within the ranges that can be achieved by this restricted physiological mechanism at the adipocyte level.

This work was supported by American Heart Association Scientist Development Grant 9630120N (to R. L. Mynatt) and National Institute of Diabetes and Digestive and Kidney Diseases Grant R01 DK-52968-02 to (J. M. Stephens).

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