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It is well recognized that the agouti/melanocortin system is an important regulator of body weight homeostasis. Given that agouti is expressed in human adipose tissue and that the ectopic expression of agouti in adipose tissue results in moderately obese mice, the link between agouti expression in human adipose tissue and obesity/type 2 diabetes was investigated. Although there was no apparent relationship between agouti mRNA levels and BMI, agouti mRNA levels were significantly elevated in subjects with type 2 diabetes. The regulation of agouti in cultured human adipocytes revealed that insulin did not regulate agouti mRNA, whereas dexamethasone treatment potently increased the levels of agouti mRNA. Experiments with cultured human preadipocytes and with cells obtained from transgenic mice that overexpress agouti demonstrated that melanocortin receptor (MCR) signaling in adipose tissue can regulate both preadipocyte proliferation and differentiation. Taken together, these results reveal that agouti can regulate adipogenesis at several levels and suggest that there are functional consequences of elevated agouti levels in human adipose tissue. The influence of MCR signaling on adipogenesis combined with the well-established role of MCR signaling in the hypothalamus suggest that adipogenesis is coordinately regulated with food intake and energy expenditure. Diabetes 52:2914–2922, 2003

Over the past decade, it has become apparent that the agouti/melanocortin system is a critical component of several biological pathways, including body weight homeostasis. Many genetic and pharmacological studies have shown that agouti and agouti-related protein (AGRP) compete with pro-opiomelanocortin (POMC)-derived peptides for binding sites on melanocortin receptors (MCRs) to regulate food intake and energy expenditure. Altered expression levels of agouti (1,2), AGRP (3,4), MC3-R (5) and MC4-R (6), and POMC (7) have been shown to disrupt the control of body weight in mice. However, to date, there have been no published observations implicating agouti, per se, in human obesity or type 2 diabetes.

The mouse and human agouti proteins are structurally similar, but the expression pattern of the mouse and human agouti genes is very different. The transcription of the wild-type mouse agouti is temporally regulated, being expressed solely in the skin during part of the hair growth cycle (1). Human agouti is expressed in diverse tissues: adipose tissue, testis, heart, liver, kidney, ovary, and skin (8,9). The divergence in expression patterns between mice and humans strongly suggests that agouti may have functions other than pigmentation in humans. The presence of agouti, and MCRs, in human adipose tissue raises questions as to their normal function in adipose tissue and whether dysregulation of agouti in adipose tissue might be associated with an obese, insulin-resistant, or diabetic phenotype.

In the original human agouti cloning study by Kwon et al. (8), an RT-PCR analysis of RNA obtained from subcutaneous fat from three normal females revealed agouti expression in two of the three fat samples and suggested that agouti might be differentially regulated in humans. More recently, agouti mRNA was found to be negatively correlated with BMI in men but not in women (10). In our recent studies, we engineered transgenic mice to overexpress agouti in adipose tissue in order to study the potential biological role of agouti/melanocortin signaling in human adipose tissue (11). The aP2-agouti transgenic mice become significantly heavier than nontransgenic littermates by 8–10 weeks of age and have almost a twofold increase in fat mass compared with nontransgenic mice (12). Moreover, there were no detectable changes in food intake in the aP2-agouti mice, suggesting changes in energy utilization and/or nutrient partitioning (13).

In the present study, expression levels of human agouti and MCRs were studied in human mesenchymal stem cells as they were differentiated into lipid-storing adipocytes. Having established the presence of both agouti and MCRs in human adipose tissue, agouti expression levels were measured in patients with a wide range in BMI and in patients with type 2 diabetes. Additionally, experiments with transgenic mice that express agouti in adipose tissue.
and isolated preadipocytes were used to determine whether there was a possible functional role of the agouti/melanocortin system in adipose tissue. Several interesting findings have emerged from these experiments. The data demonstrate that human agouti expression is upregulated by glucocorticoids and that agouti and the cortisol-producing enzyme 11β hydroxysteroid dehydrogenase type 1 (11β-HSD-1) are upregulated in patients with type 2 diabetes. Third, we find that the agouti/melanocortin system is a potent and direct regulator of adipogenesis.

**RESEARCH DESIGN AND METHODS**

**Human mesenchymal cell differentiation.** Cells (BioWhittaker) were plated at a density of 2-5 × 10^3/cm^2 and grown at 37°C in an atmosphere of 95% air and 5% CO₂, as previously described (14). Cells were grown to 100% confluence in the growth medium supplemented with 5% fetal bovine serum (FBS). Five-day postconfluent cells were incubated in adipogenesis induction medium (AIM-M199; 1 mM dexamethasone, 0.2 mM indomethacin, 170 mM/μl insulin, 0.5 mM/μl isobutyl-methylxanthine [IBMX], 10% FBS, 0.05 units/ml penicillin, and 0.05 μg/ml streptomycin) for 3 days. Next, the cells were incubated for 1 day in adipogenesis maintenance medium (AMM-M199; 170 μM/μl insulin, 10% FBS, 0.05 units/ml penicillin, and 0.05 μg/ml streptomycin) and then switched to AIM for 3 days. After a third exposure to AIM, cells were maintained in AMM for up to 21 days after the initiation of differentiation.

**Isolation and differentiation of mouse preadipocytes.** Epidydimal and inguinal fat pads, collected from 19- to 21-day-old FVB, β-actin promoter-agouti (BAP-agouti), and α2P-agoni mice, were minced and digested with collagenase class I (2 mg/ml, Worthington Biochemical) in a shaking bath for 1 h at 37°C. Dissociated cells were filtered through a 100 μm/μl cell strainer (Becton Dickinson Labware) and separated by centrifugation at 360g for 5 min. Pelleted preadipocyte cells were resuspended for 1 min. in erythrocyte lysis buffer (Sigma) to remove erythrocyte contamination and centrifuged at 360g for 5 min. Cells were plated in 35-mm Petri dishes (purchased through Life Technologies) or in complete medium. Subconfluent primary cultures were trypsinized and replated at a density of 2 × 10^4 cells/cm^2 in a 24-well culture plate and kept in complete medium. At confluence, cells received differentiation medium (DMEM/F12 medium (Invitrogen Life Technologies) supplemented with 15% of FBS) and maintenance medium (DMEM/F12 with 5% FBS supplemented with 0.5% insulin, 10% FBS, 5% dexamethasone, and 0.5% penicillin/0.5% streptomycin) and then switched to AIM for 3 days. After a third exposure to AIM, cells were maintained in AMM for up to 21 days after the initiation of differentiation.

**RNA isolation and RT-PCR analysis.** Total RNA was isolated by the Trizol method (Life Technologies) and applied to Rneasy columns (Qiagen). The quality was verified by visualizing on a gel. For nonquantitative RT-PCR, 1 μg total RNA was used for the cDNA synthesis and 1:40 of that reaction was used in 25 μl of PCR reaction. The following primer sets were used. Human (forward, reverse primer): agouti (GTGCTCTATGGGCGCTGGA, GGAAGAAGGCAGC), MC1-R (GGAGATCTTGGGCTTCA, GGAAGAAGGCAGC), MC2-R (ATCCCTTGCGTTGAGAGCTG), MC4-R (GCGGGTCGGGATCAGGCA, probe 6-FAM-CATCTACGGTGAGC), MC5-R (TACACTGGTGTCGCTGCA, probe 6-FAM-GGAGTCTCCATGTTGGA), and AGTCTCCTA). Mouse (forward, reverse primer): MC1-R (CACCAGGACGGAGGAA, CCAAGAGACGGCAGCA), MC2-R (CTCTGGGCGCTGCGTCA, GGAAGAAGGCAGC), MC3-R (CTCTGGGCGCTGCGTCA, AGAGTCTGGGCGCTGGA), MC4-R (CACCAGGACGGAGGAA, probe 6-FAM-CATCTACGGTGAGC). The Taqman probe primer system (Applied Biosystems) was used for quantitative RT-PCR. Primer/probe sets are as follows. Agouti: forward CAACTCCGTCCACCTAAGGGT, probe 6-FAM-CCCTTCTGCTCTAATGGGCTGCCAC. The primer/probe sequences were as follows. Agouti: forward CAACTCCGTCCACCTAAGGGT, probe 6-FAM-CCCTTCTGCTCTAATGGGCTGCCAC. The primer/probe sequences were as follows. Agouti: forward CAACTCCGTCCACCTAAGGGT, probe 6-FAM-CCCTTCTGCTCTAATGGGCTGCCAC. The primer/probe sequences were as follows. Agouti: forward CAACTCCGTCCACCTAAGGGT, probe 6-FAM-CCCTTCTGCTCTAATGGGCTGCCAC. The primer/probe sequences were as follows. Agouti: forward CAACTCCGTCCACCTAAGGGT, probe 6-FAM-CCCTTCTGCTCTAATGGGCTGCCAC. The primer/probe sequences were as follows. Agouti: forward CAACTCCGTCCACCTAAGGGT, probe 6-FAM-CCCTTCTGCTCTAATGGGCTGCCAC. The primer/probe sequences were as follows. Agouti: forward CAACTCCGTCCACCTAAGGGT, probe 6-FAM-CCCTTCTGCTCTAATGGGCTGCCAC. The primer/probe sequences were as follows. Agouti: forward CAACTCCGTCCACCTAAGGGT, probe 6-FAM-CCCTTCTGCTCTAATGGGCTGCCAC. The primer/probe sequences were as follows. Agouti: forward CAACTCCGTCCACCTAAGGGT, probe 6-FAM-CCCTTCTGCTCTAATGGGCTGCCAC. The primer/probe sequences were as follows. Agouti: forward CAACTCCGTCCACCTAAGGGT, probe 6-FAM-CCCTTCTGCTCTAATGGGCTGCCAC. The primer/probe sequences were as follows. Agouti: forward CAACTCCGTCCACCTAAGGGT, probe 6-FAM-CCCTTCTGCTCTAATGGGCTGCCAC. The primer/probe sequences were as follows. Agouti: forward CAACTCCGTCCACCTAAGGGT, probe 6-FAM-CCCTTCTGCTCTAATGGGCTGCCAC. The primer/probe sequences were as follows. Agouti: forward CAACTCCGTCCACCTAAGGGT, probe 6-FAM-CCCTTCTGCTCTAATGGGCTGCCAC. The primer/probe sequences were as follows. Agouti: forward CAACTCCGTCCACCTAAGGGT, probe 6-FAM-CCCTTCTGCTCTAATGGGCTGCCAC. The primer/probe sequences were as follows. Agouti: forward CAACTCCGTCCACCTAAGGGT, probe 6-FAM-CCCTTCTGCTCTAATGGGCTGCCAC. The primer/probe sequences were as follows. Agouti: forward CAACTCCGTCCACCTAAGGGT, probe 6-FAM-CCCTTCTGCTCTAATGGGCTGCCAC. The primer/probe sequences were as follows. Agouti: forward CAACTCCGTCCACCTAAGGGT, probe 6-FAM-CCCTTCTGCTCTAATGGGCTGCCAC. The primer/probe sequences were as follows. Agouti: forward CAACTCCGTCCACCTAAGGGT, probe 6-FAM-CCCTTCTGCTCTAATGGGCTGCCAC.
human cells or fat. A critical problem with RT-PCR detection of MCR mRNA is that the MCR genes are intronless. Therefore only a small amount of genomic DNA contamination in the RNA preparation will give a false-positive. It is unclear if the human RNA purchased for the previous study (15) was DNase treated for the RT-PCRs. Hence, it is possible that the commercial RNA may have been contaminated with genomic DNA.

RT-PCR analysis detected both MC2-R and MC5-R in murine 3T3-L1 cells (Fig. 1B) as they differentiated into adipocytes, an observation which is consistent with a previous study on the MCR subtype expression in 3T3-L1 adipocytes (16). None of the other MCR subtypes were detectable in 3T3-L1 cells. MC2-R and MC5-R were detectable in FVB/N mouse adipose tissue, and they were found primarily in the adipocyte fraction. MC1-R, MC2-R, and MC5-R were found in mouse preadipocyte culture (Fig. 1C).

To examine the agouti expression pattern during human adipocyte differentiation, human mesenchymal stem cells were cultured to 95% confluence and induced to differentiate as described in RESEARCH DESIGN AND METHODS. We observed that the expression of agouti mRNA increased through each cycle of exposure to the differentiation media and declined when the cells were returned to basal media (Fig. 2A and B). The decline in agouti expression that occurred when cells were returned to the basal media suggested that individual components of the differentiation media were capable of regulating agouti expression. We observed that the addition of dexamethasone to either confluent mesenchymal stem cells or differentiated adipocytes increased agouti expression to the same level observed with the complete differentiation cocktail (Fig. 2C). These in vitro results indicate that glucocorticoids upregulate agouti expression in human adipocytes.

Since transgenic mice that express agouti in adipose tissue become obese (12), agouti mRNA was quantitated in the subcutaneous fat from humans with a broad range of BMIs. There was no correlation between agouti expression levels and BMI in either men or women (Fig. 3A). However, agouti mRNA was significantly greater in women than in men (Fig. 3B). Agouti mRNA was also measured in individuals with type 2 diabetes with body fat similar to moderately obese people (Table 1 and Fig. 3A). There were significantly higher levels of agouti expression in diabetic subjects than in nondiabetic individuals (Table 1 and Fig. 3B). The data in Fig. 3A demonstrate that type 2 diabetic subjects have higher agouti levels than nondiabetic subjects across a range of BMIs. Again there was a
sex effect, with diabetic women having the highest overall agouti expression levels (Fig. 3B).

Cell culture experiments with either confluent preadipocytes or differentiated adipocytes demonstrated that glucocorticoids potently increase agouti expression. While elevated glucocorticoid levels may play a significant role in the development of obesity and type 2 diabetes (17), there are no definitive reports linking elevated circulating glucocorticoid levels to the obese/diabetic state. Additionally, we found no relationship between cortisol secretion (as measured by salivary cortisol) and agouti mRNA (data not shown). However, the enzyme 11β-HSD-1 can regulate intracellular glucocorticoid concentrations by regenerating active glucocorticoid (cortisol in humans, corticosterone in mice) from inactive cortisone and 11-dehydrocorticosterone. We observed that 11β-HSD-1 mRNA levels were significantly higher in the same cohort of diabetic subjects that had elevated agouti expression levels (Fig. 3C), demonstrating another correlation between glucocorticoids and agouti.

Having observed the elevated agouti levels in diabetic humans, transgenic mice and cell culture experiments were used to determine whether there were functional consequences of agouti expression in adipose tissue. Microarray data from 3-week-old aP2-agouti transgenic mice suggested a relationship between agouti expression and preadipocyte hyperplasia (data not shown). Therefore, we measured the DNA content from the epididymal fat pads of aP2-agouti transgenic mice to determine cell density. Both total DNA and DNA per milligram fat were significantly higher in the transgenic mice at 4 weeks of age, indicating that more preadipocytes were present in the fat pads of aP2-agouti mice (Fig. 4A). The increased amount of DNA in the fat of aP2-agouti mice led us to examine the effects of agouti and αMSH on preadipocyte proliferation in vitro. We observed that αMSH inhibited proliferation in both mouse and human preadipocytes (Fig. 4B and C). Our data also indicate that agouti has a slight stimulatory effect on proliferation, but it is clear that agouti blocked the inhibitory effects of αMSH. Interestingly, AGRP was not able to block the inhibitory effects of αMSH on proliferation (data not shown). Forskolin was also a potent inhibitor of proliferation, but agouti was not able to block the inhibitory effects of forskolin (data not shown). These data, taken together with the increased DNA content at 4 weeks of age in the aP2-agouti mice, suggest that αMSH and agouti regulate adipocyte proliferation in a reciprocal fashion.

Several studies have shown that PPARγ is an essential transcription factor for differentiation and maturation of adipocytes (18–24). Additionally, ectopic expression of PPARγ in nonprecursor fibroblast cells promotes lipid accumulation and confers the characteristics of mature adipocytes (25). Recent studies have also suggested that the STAT (signal transducers and activators of transcription) family of transcription factors may also be important in fat cells (26–29). We have previously shown that both agouti treatment of 3T3-L1 adipocytes and agouti expres-
sion in mouse adipose tissue increases STAT1, STAT3, and PPARγ protein levels (13). Since PPARγ is a key regulator of adipocyte differentiation and agouti induces PPARγ, we initiated experiments to address the ability of agouti to influence adipocyte differentiation. The levels of differentiation were measured by lipid accumulation in nontransgenic and BAP-agouti transgenic mice (30). Cells from the nontransgenic and BAP-agouti transgenic mice were included in this study because the B-actin promoter will drive agouti expression in the preadipocytes. However, preadipocytes isolated from the aP2-agouti mice should not express agouti because the aP2 promoter is not active until late in adipocyte differentiation. Preadipocytes were isolated from 19- to 21-day-old mice by collagenase digestion and centrifugation. The preadipocytes were plated and grown to confluence and induced to differentiate as described in RESEARCH DESIGN AND METHODS. After 5 days of differentiation, the cells were stained with Oil Red-O to measure lipid accumulation. Only 20% of the cells from wild-type mice had significant lipid accumulation (Fig. 5A). However, both the addition of 10 nmol/l COOH-terminal agouti peptide to wild-type preadipocytes (Fig. 5B) and the ectopic expression of agouti (Fig. 5C) caused a significant increase in Oil Red-O staining. After photography, the Oil Red-O was extracted from the adipocytes and quantified (Fig. 5D), further demonstrating that agouti increases adipocyte differentiation.

Table 1 provides a summary of the expression of agouti and 11β-HSD-1 mRNA in human subcutaneous adipose tissue. Agouti and 11β-HSD-1 mRNAs were measured as described in RESEARCH DESIGN AND METHODS and normalized to cyclophilin mRNA. A: There was no relationship between BMI and agouti mRNA, but agouti (B) and 11β-HSD-1 (C) mRNA are increased in diabetes. T2DM, type 2 diabetes.

**DISCUSSION**

The hypothalamic melanocortin system is an important regulator of body weight and appetite. The aim of these studies was to understand the role of the melanocortin system in adipose tissue. These studies reveal for the first time that expression of agouti mRNA is increased in diabetes, but not obesity, and that glucocorticoids upregulate agouti mRNA in vitro. They also reveal that there is increased expression of the cortisol-activating enzyme 11β-HSD-1 in diabetes. We also provide compelling data...
on the functional consequences of local agouti production; αMSH inhibits proliferation of human and murine preadipocytes, and agouti blocks this effect. Furthermore, agouti enhances differentiation of murine and human preadipocytes in vitro. Combined with previous data demonstrating that agouti upregulates PPARγ protein levels, this suggests that agouti is a paracrine factor in adipose tissue that can regulate both proliferation and differentiation of preadipocytes.

In addition to the effects of agouti on adipogenesis, there is considerable precedence for the regulation of mature adipocyte metabolism by agouti and POMC-derived peptides. The mRNA levels for fatty acid synthase and stearoyl-CoA desaturase, two key enzymes in de novo fatty acid synthesis and desaturation, respectively, were dramatically increased in obese (A<sup>V</sup>) mice relative to lean (a/a) controls (31). Additionally, treatment of fully differentiated 3T3-L1 adipocytes with recombinant agouti protein increased fatty acid synthase and stearoyl-CoA desaturase mRNA levels (31). A potential mechanism for the increased fatty acid synthase and stearoyl-CoA desaturase levels is that they are transcriptional targets of PPARγ. In opposition to the adipogenic effects of agouti, ACTH, αMSH, and β-LPH are potent lipolytic hormones, but considerable interspecies variability exists in the lipolytic response of adipocytes to melanocortins (32). The negative effects of melanocortins on adipogenesis (inhibition preadipocyte proliferation and increased lipolysis), combined with the positive effects of agouti on adipogenesis (increased PPARγ and differentiation), strongly suggest a coordinate control of the adipogenesis.

Agouti appears to antagonize αMSH and ACTH stimula-
tion of cAMP at all five of the MCRs (33,34). αMSH stimulates cAMP levels at all the MCRs except MC2-R, and ACTH stimulates cAMP levels at all the MCRs (33). Previous studies demonstrated that mouse adipocytes express high-affinity binding sites for melanocortin peptides (35) and that MC1-R, MC2-R, and MC5-R are expressed in adipocytes and adipose tissue (16,36). In these studies, high expression levels of MC1-R and low levels of MC2-R were observed in the human preadipocytes (Fig. 1), and based on the inactivity of αMSH at MC2-R, it is logical to suggest that MC1-R is responsible for the antiproliferative effects of αMSH in preadipocytes.

It should be noted that the inhibitory effects of αMSH cellular proliferation are not restricted to preadipocytes. One of the hallmark phenotypes of the yellow obese syndrome is neoplasia and an increased incidence of tumors (reviewed by Wolff et al. [37] and Yen et al. [38]). Additionally, treatment of melanocytes with αMSH promotes terminal differentiation, resulting in an inhibition of proliferation, and agouti reverses αMSH effects and inhibits pigmentation and promotes proliferation (39–41).

We have previously shown that agouti increases PPARγ expression in fully differentiated 3T3-L1 adipocytes and in mice that ectopically express agouti in adipose tissue (12,13). The present study demonstrates that agouti stimulates adipocyte differentiation. Once again, the mechanism appears to be mediated through PPARγ, since PPARγ mRNA levels are as high in the confluent undifferentiated preadipocytes from agouti-expressing mice, as compared with the fully differentiated adipocytes.

Perhaps the most clinically relevant findings are the upregulation of human agouti by glucocorticoids and the elevated levels of agouti and 11β-HSD-1 mRNA in subcutaneous fat from patients with type 2 diabetes. Taken together, these data suggest a model where by the hyperglycemia of diabetes activates 11β-HSD-1 transcription and increased local production of cortisol, which then increases agouti mRNA. An alternate explanation is that a common factor induces both agouti and 11β-HSD-1 transcription. Given that dexamethasone potently upregulates agouti in vitro, we hypothesize that the paracrine model depicted in Fig. 6 is a more probable explanation and warrants further investigation. 11β-HSD-1 causes visceral obesity when overexpressed in transgenic mice (42), but this effect is unlikely to be due to the upregulation of agouti, as murine adipose tissue does not express agouti. On the other hand, activity of 11β-HSD-1 in human adipose tissue is increased in visceral adipose tissue and has been
proposed as a cause of central adiposity (43). Our data suggest that agouti might play a role in central obesity as a downstream target of glucocorticoids. Cortisol might be produced locally via 11β-HSD-1 or the result of an overactive hypothalamic-pituitary-adrenal axis (44,45). Either way, increased exposure of adipose tissue to cortisol might serve as a stimulus for adipose tissue proliferation and differentiation, and these data suggest that the autocrine/paracrine production of agouti is a putative mediator of this effect.

When combined with previous studies in transgenic mice overexpressing agouti, the functional implications of increased agouti protein in adipose tissue are clear: agouti increases both proliferation (by blocking the effects of αMSH) and differentiation (through the upregulation of PPARγ protein).

In conclusion, mRNA levels were significantly elevated in subjects with type 2 diabetes. The regulation of agouti in cultured human adipocytes revealed that insulin did not regulate agouti mRNA, whereas dexamethasone treatment potently increased the levels of agouti mRNA. This finding, as recapitulated in vivo in humans, where increased expression of the cortisol-activating enzyme 11β-HSD-1 was upregulated in concert with agouti in type 2 diabetes. Experiments with cultured human preadipocytes and with cells obtained from transgenic mice that overexpress agouti demonstrated that MCR signaling in adipose tissue regulates both preadipocyte proliferation and differentiation. Taken together, these results reveal that agouti can regulate adipogenesis at several levels and suggest that there are functional consequences of elevated agouti levels in human adipose tissue. The influence of MCR signaling on adipogenesis combined with the well-established role of MCR signaling in the hypothalamus suggest that adipogenesis is coordinately regulated with food intake and energy expenditure.

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