Regulation of leptin by agouti

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Claycombe, Kate J., Bing Zhong Xue, Randall L. Mynatt, Michael B. Zemel, and Naima Moustaid-Moussa. Regulation of leptin by agouti. Physiol Genomics 2: 101–105, 2000.—Dominant mutations at the mouse Agouti locus lead to ectopic expression of the Agouti gene and exhibit diabetes, obesity, and yellow coat color. Obese yellow mice are hyperinsulimic and hyperleptinemic, and we hypothesized that Agouti directly induces leptin secretion. Accordingly, we used transgenic mice expressing agouti in adipocytes (under the control of aP2 promoter, aP212) to examine changes in leptin levels. Agouti expression in adipose tissue did not significantly alter food intake, weight gain, fat pad weight, or insulinemia; however, the transgenic mice were hyperglycemic. We demonstrated that plasma leptin levels are approximately twofold higher in aP212 transgenic mice compared with their respective controls, whereas ubiquitous expression of agouti (under the control of β-actin promoter, BAP20) led to a sixfold increase in leptin. Insulin treatment of aP212 mice increased adipocyte leptin content without affecting plasma leptin levels. These findings were further confirmed in vitro in 3T3-L1 adipocytes treated with recombinant Agouti protein and/or insulin. Agouti but not insulin significantly increased leptin secretion, indicating that insulin enhances leptin synthesis but not secretion while Agouti increases both leptin synthesis and secretion. This increased leptin synthesis and secretion was due to increased leptin mRNA levels by this protein. Increased adipocyte leptin content by this protein and/or insulin was independent of agouti-melanocortin receptor antagonism. Furthermore, recent reports demonstrated that leptin effects on food intake require functional MC4-R (29). However, to date, no studies addressed peripheral actions of Agouti on leptin secretion.

Leptin, the obese (ob) gene product, is produced primarily by adipose cells (7). Leptin plays a key role in regulation of food intake and energy balance (8, 9) primarily by binding to hypothalamic leptin receptors. In addition, leptin exerts direct metabolic effects in peripheral tissues including adipose tissue (29). Changes in adipose tissue mass generally correlate with changes in plasma leptin levels (8, 16), which are elevated in several obese animal models, including yellow agouti mice and humans (8, 19).

Pro-opiomelanocortin (POMC) serves as a precursor to α-MSH which is an endogenous ligand for melanocortin receptors 3 and 4 (MC3-R and MC4-R) in the hypothalamus (18, 26). Agouti and agouti-related protein (AGRP) antagonism at the melanocortin receptors MC3-R and MC4-R (26) have been suggested to mediate agouti-induced hyperphagia (10, 26). A limited number of studies demonstrated agouti-leptin interaction in central nervous system (CNS). Leptin has been shown to stimulate hypothalamic POMC gene expression (28), possibly limiting Agouti effects via MSH antagonism. Furthermore, recent reports demonstrated that leptin effects on food intake require functional MC4-R (29). However, to date, no studies addressed peripheral actions of Agouti on leptin secretion.

We have previously shown that Agouti regulates adipocyte metabolism including lipogenesis and lipolysis (12, 31). However, it is not known whether agouti regulates leptin. Accordingly, we addressed this issue using transgenic mice that express agouti under the adipocyte-specific promoter aP2, as well as in cultured 3T3-L1 adipocytes. These models allow us to examine Agouti effects on adipocyte metabolism independent of its central effects on feeding behavior. Our data demonstrate that expression of agouti in adipose tissue increased adipose tissue leptin levels and plasma leptin, whereas administration of insulin enhanced leptin synthesis but not secretion. Furthermore, in vitro studies performed in murine 3T3-L1 adipocytes further supported these differential in vivo effects of agouti and insulin on leptin secretion. Finally, increased leptin synthesis and secretion by Agouti was due to increased leptin mRNA content by this protein and was independent of agouti-melanocortin receptor antagonism.
MATERIALS AND METHODS

Transgenic mice, BAP20 or aP212 transgenic mice, which express agouti ubiquitously under the β-actin (BAP) or the adipocyte-specific aP2 promoter (aP2), respectively, were generated as previously described (14, 22). Transgenic mice used in these studies were maintained in our breeding colony at the Department of Nutrition, University of Tennessee. All mice were weaned at 4 wk of age and were fed a diet containing 11% fat by weight (Mouse Diet 5015; Purina). Food and water were provided ad libitum. All data are from mice that are homozygous for the transgene, and nontransgenic littermates were used as controls. The presence of agouti mRNA was confirmed by Northern blot hybridization using total RNA isolated from various tissues. In aP212 mice, agouti mRNA was detected only in adipose tissue (22), whereas agouti was expressed in all tissues from BAP20 mice (14).

Insulin injection. Twelve-week-old male aP212 line mice were subcutaneously injected with human insulin (Eli Lilly) at a daily dose of 1 U/day per mouse for 2 wk as we previously reported (22). Insulin (100 U/ml) was diluted prior to daily injection with PBS to make final injection volume of 200 µl (1 U). The systemic effect of injected insulin was confirmed by measuring blood glucose levels. The blood glucose levels decreased significantly (immediately following subcutaneous injection with PBS to make final injection volume of 200 µl (1 U)). The systemic effect of injected insulin was confirmed by measuring blood glucose levels. The blood glucose levels decreased significantly (immediately following subcutaneous injection) and normalized within 4 h after the injection (data not shown) in both transgenic and in control mice. Prior to tissue collection, mice were anesthetized using pentobarbital sodium, and blood was obtained by cardiac puncture. Adipose tissues (epididymal, perirenal, abdominal, and subscapular) were removed, weighed, and quickly frozen in liquid nitrogen until use. All of the above protocols were approved by Institutional Animal Care and Use Committee of the University of Tennessee in Knoxville.

Plasma leptin levels. Blood was collected via heart puncture using heparinized tubes. Plasma was prepared, and 100 µl was used in a radioimmunoassay (RIA) using a kit purchased from Linco (St. Charles, MO) to determine leptin levels. Similarly, leptin levels were measured in culture media from 3T3-L1 cells as indicated above for plasma leptin. Adipose tissue was homogenized in PBS, and 100 µl of the homogenized tissue extract was used in the RIA to determine intracellular leptin levels, which were then corrected to protein content in the extracts; protein content was assayed using the Bradford method (2).

Cell culture. 3T3-L1 cells were grown and differentiated as previously described (13, 21). Briefly, cells were grown to confluence in standard medium (DMEM supplemented with 10% FBS). Adipocyte differentiation was induced by treating confluent cells with dexamethasone (250 nM) and isobutylmethylxanthine (0.5 mM). Cells were then maintained for three additional days in standard medium, then changed to serum-free medium (containing 1% BSA) followed by treatment with Agouti protein and/or insulin, MTII (melanocortin receptor agonist) and SHU-9119 (MC4-R antagonist) as indicated in the legends of Figs. 4 and 5.

RNA analysis. Total RNA was isolated from 3T3-L1 adipocytes treated with or without 50 nM Agouti, using the guanidium thiocyanate/CsCl purification method as we previously described (21). These RNAs were then subjected to agarose gel electrophoresis, transferred to nylon membranes, then hybridized with ob and actin cDNAs. Autoradiograms are quantified by densitometry scanning, and the data for ob mRNA were normalized to those for actin mRNA.

RESULTS

Effect of ubiquitous and adipocyte-specific agouti expression on plasma leptin levels. Recent studies have shown that obese yellow mouse exhibited high levels of plasma leptin levels (8). In agreement with these studies, transgenic mice ubiquitously expressing agouti expressed five to sixfold higher plasma leptin levels compared with controls (P < 0.0005) (Fig. 1). Transgenic mice expressing agouti only in adipose tissue (aP212) also exhibit a twofold increase in plasma leptin levels compared with controls (Fig. 1, P < 0.05). Similar plasma leptin levels were obtained in another aP2 transgenic line (data not shown), as demonstrated in aP212 mice. Several reports including from our laboratory have previously documented that BAP20 mice exhibit significantly higher body weight, fat pad weight, and circulating leptin and insulin levels (14, 33). Although these parameters are comparable between control and aP212 transgenic mice (Table 1), both aP2 (Table 1) and BAP20 (33) are hyperglycemic. These studies demonstrate that adipocyte-specific and ubiquitous expression of agouti lead to increased circulating levels of leptin. Furthermore, the effects of adipocyte expression in aP2 mice are independent of food intake, insulin levels, or weight/fat gain.

Effects of insulin injection on plasma and adipocyte leptin levels in aP2 transgenic mice. Daily injection of insulin to aP212 transgenic mice or to nontransgenic controls did not affect plasma leptin levels (Fig. 2). This suggests that, regardless of the genotype, insulin did not affect plasma leptin levels. When data with and without insulin treatment were combined for each genotype (controls and transgenic), a significant difference in the plasma leptin level was observed due to the genotype differences (data not shown).

Statistical analysis. Multiple and nested analyses of variance (SAS, Cary, NC) were used to compare the data. All data are expressed as means ± SE at a significance level of 0.05.
Interestingly, although insulin treatment did not result in changes in plasma leptin levels, administration of insulin to aP2 transgenic mice resulted in a significant increase in adipose tissue leptin levels (Fig. 3). However, insulin only slightly but not significantly increased leptin synthesis in control mice (Fig. 3). Taken together, these results demonstrate that Agouti increases both leptin synthesis and secretion, whereas insulin increases leptin synthesis (significantly in transgenic mice) without modifying its secretion. In agreement with increased plasma leptin levels in transgenic compared with control mice (Fig. 1), adipose tissue leptin levels were also significantly higher in transgenic vs. control mice (Fig. 3, controls vs. transgenics and adipose tissue in control mice vs. transgenic mice). This indicates that agouti increases both leptin synthesis and secretion.

Effects of agouti on leptin secretion and ob mRNA content in 3T3-L1 adipocytes. Differential effects of Agouti and insulin on secreted leptin were further examined using 3T3-L1 adipocytes. Leptin levels in culture media collected from 3T3-L1 adipocytes treated with Agouti, insulin or a combination of Agouti and insulin are shown in Fig. 4A. These in vitro data confirm the in vivo observations. Agouti significantly increased secreted leptin levels (0.93 ± 0.12 vs. 0.58 ± 0.11 ng/ml, P < 0.05), whereas insulin was without effect (0.51 ± 0.05 ng/ml). Addition of Agouti and insulin caused a significant increase (1.0 ± 0.17 ng/ml) similar to that caused by Agouti alone (Fig. 4A). To determine the mechanism of increased leptin secretion, we investigated the effects of Agouti on ob mRNA. In agreement with Agouti induction of leptin secretion, data in Fig. 4B demonstrate that agouti also significantly increased ob mRNA content, indicating a pre-translational regulation of the ob gene by Agouti. Since agouti-induced obesity has been postulated to be mediated by agouti-melanocortin receptor antagonism at MC4-R, we tested this possibility in 3T3-L1 adipocytes. As indicated in Fig. 5, agouti significantly increases leptin secretion as demonstrated above, whereas neither melanocortin receptor agonist MTII nor MC4-R antagonist SHU-9119 significantly affected leptin secretion. Furthermore, none of these drugs changed agouti-induced leptin secretion. These data indicate that Agouti stimulates leptin secretion via an MC4-R-independent mechanism.

**DISCUSSION**

Several recent studies have investigated insulin regulation of leptin, but inconsistent results were reported. Some investigators have suggested that insulin treatment significantly increased leptin secretion (1, 27), whereas others showed no significant effect of elevated insulin levels on plasma leptin content (15, 23, 25). Pharmacological doses (700 nM) of insulin were used to
induce leptin mRNA and plasma levels, in vitro (1); however, the effects of this high insulin concentration might be mediated through insulin-like growth factor-1 receptors. Insulin is known to increase plasma leptin levels (Fig. 2), may have resulted from increased clearance of circulating leptin or its binding to other proteins. It is possible that insulin may have an effect on packaging and secretory processes of leptin; although this is not within the scope of this work, it merits further investigation. Our studies demonstrate an effect of insulin on adipose tissue leptin without any changes in plasma leptin. This indicates a lack of correlation between changes in plasma and adipocyte intracellular levels of leptin. In agreement with these findings, a recent study found no significant relationship between ob mRNA content and plasma leptin or adiposity (24).

Previous studies demonstrated that yellow obese mice exhibit elevated levels of plasma leptin (8). Similarly, BAP20 transgenic mice, which recapitulate the yellow mouse obesity syndrome (14), also express very high levels of plasma leptin (Ref. 33 and Fig. 1). This increase in plasma leptin is paralleled by a significant increase in adiposity in these models (7, 8, 19). Although, aP212 transgenic mice exhibit approximately a twofold increase in plasma leptin levels compared with their control littermates, body weights, fat pad weights, and food intake were similar between controls and transgenics. This increased leptin independent of obesity may be indicative of leptin resistance and is of interest since recent studies on Japanese Americans (6) showed that increased leptin levels are associated with subsequent gain of weight and adiposity and suggested that obesity in this population is associated with leptin resistance and is preceded by increased leptin levels. Long-term studies on aP2 mice will be useful to determine whether obesity preceded by hyperleptinemia develops at a later age. Recent reports indicated that hyperleptinemia may be an early marker of juvenile obesity (4). Since aP2 mice do not express agouti in the brain and since secreted Agouti acts locally and does not circulate, it is possible that increased levels of leptin in these mice may play a role in limiting and/or delaying agouti-induced adiposity in aP212. Our studies thus demonstrate that agouti regulation of adipocyte metabolism may play a key role in the agouti obesity syndrome. The effect of agouti expression in adipose tissue on leptin secretion was further confirmed in 3T3-L1 adipocytes where Agouti increased both leptin secretion and ob mRNA content, indicating that Agouti regulates leptin at the pretranslational level. The molecular mechanisms of this regulation merit further investigation. Agouti-induced obesity is in part due to Agouti (or AGRP)-MSH antagonism at the MC4-R leading to hyperphagia. MC4-R is primarily expressed in the brain; however, it is also expressed in adipose tissue (5). Our studies in 3T3-L1 adipocytes however, demonstrate that these receptors are not involved in agouti-induced leptin secretion.

In summary, our current findings demonstrate that Agouti directly upregulates adipose tissue leptin as well as plasma leptin and ob mRNA levels. Since agouti is normally expressed in human adipose tissue (17), aP2 transgenic mice are useful models to study the role of agouti in adipocyte metabolism. Our present data indicate that agouti may function as an autocrine regulator of leptin in human adipocytes. This is the first report of regulation of an obesity gene product by another obesity gene product, suggesting that interaction between obesity genes may play a key role in obesity syndromes.
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