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I am submitting herewith a dissertation written by Kate J. Claycombe entitled "Mechanisms of agouti-induced obesity: Effects on adipocyte metabolism and interaction with insulin". I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirement for the degree of Doctor of Philosophy, with a major in Human Ecology.

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MECHANISMS OF AGOUTI-INDUCED OBESITY: EFFECTS ON ADIPOCYTE METABOLISM AND INTERACTION WITH INSULIN

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Kate J. Claycombe
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DEDICATION

I dedicate this dissertation to

my husband Wayne Claycombe and my mother Maria N. Joh.

Their loving support, patience and encouragement enabled me to complete this work.

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I am indebted to Dr. Naima Moustaid Moussa. Her patience, critical insight and sincere interest in the student make her truly exemplary of the title "mentor and teacher". She devoted an immeasurable amount of time for helpful suggestions in the progress and completion of this dissertation. Many special thanks also go to future Dr. Hanna Moussa, for his help and support.

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Most importantly, sincere appreciation is given to my husband Wayne Claycombe. Completion of this work would not have been possible without his unconditional love. To Wayne, goes my special gratitude for his patience, encouragement, support and tolerance.

ABSTRACT

Dominant mutations at the agouti locus such as viable yellow (A^{vy}) cause a syndrome of marked obesity and diabetes in addition to a characteristic yellow coat color. Recent studies indicate that agouti acts both centrally and peripherally to induce obesity. We hypothesized that agouti modulation of adipocyte metabolism may account for part of the yellow mouse obesity. Studies from our laboratory indicated that agouti increases adipocyte de novo lipogenesis and triglyceride levels in a calcium (Ca^{2+})-dependent manner. However, the precise molecular mechanisms that are involved in agouti regulation of adipocyte metabolism, have not been determined.

The objective of this work is to determine the mechanisms of agouti action on adipocyte metabolism using two specific markers of adiposity: (1) leptin, the product of the obesity gene, *ob* which is secreted by fat cells in amounts that are positively correlated with adiposity, and (2) fatty acid synthase (FAS), a key de novo lipogenic enzyme which is highly responsive to hormonal and nutritional changes.

We investigated effects of agouti and its interaction with insulin on leptin synthesis and secretion in cultured adipocytes as well as in transgenic mice overexpressing agouti in adipose tissue (under the control of adipocyte specific promoter aP2). We also investigated whether transcription rate of the FAS gene in cultured adipocytes is altered via agouti specific response elements in the FAS promoter.

Results from this study demonstrated that agouti significantly increase intracellular and plasma leptin levels in aP2 transgenic mice relative to control mice. Further, administration of insulin (1 unit/day) increased intracellular leptin levels without any significant effect on plasma leptin. The lack of insulin effect on plasma leptin levels were further confirmed by in vitro assays; media collected from 3T3-L1 adipocytes that were treated with 100 nM insulin showed no effect on leptin secreted into the culture media.

These results suggest that agouti increases leptin synthesis and secretion while insulin only modulates leptin synthesis. Agouti may increase leptin levels as a result of its effect on triglyceride storage. Alternatively, agouti may directly regulate leptin synthesis and secretion by altering *ob* gene expression. However, further studies are required to determine mechanisms of agouti and insulin regulation of leptin.

In addition to agouti regulation of leptin, our study demonstrate that both agouti and insulin upregulate FAS gene transcription. Furthermore, agouti and insulin exert additive effects on FAS gene transcription. Using transfection assays, we demonstrated that transcriptional regulation of the FAS gene by agouti was mediated by novel agouti response elements within the FAS promoter. This agouti responsive region mapped to a region distinct from the previously identified insulin responsive region. We confirmed the specificity of adipocyte nuclear factor(s) binding to this response region by electrophoretic gel mobility-shift assays. Interestingly, agouti response elements appeared to be also responsive to intracellular calcium.

In summary, the results from this study indicate that agouti effects on adipocyte may contribute to yellow mouse obesity. We demonstrated that agouti affects adipocyte metabolism by (1) inducing synthesis and secretion of leptin levels, and (2) by increasing FAS transcription rate via novel agouti responsive elements that are distinct from the previously mapped insulin response element.

Results from this investigation are relevant to human obesity. Unlike mice, humans normally express agouti in adipose tissue. As shown in aP2 transgenic mice, high levels of insulin (especially in hyperinsulinemic type II diabetic patients) combined with agouti expression in adipose tissue may contribute to increased adiposity. Therefore, modulation of agouti expression/action may be a potential target in therapeutic intervention to treat obesity and diabetes.

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PART 1

INTRODUCTION

I. OBESITY, GENERAL BACKGROUND

Obesity is defined as excess body fat. The most widely used measure of obesity is body mass index (BMI) (Table 1). BMI is calculated as weight in kilograms divided by the square of the height in meters (kg/m^2). This value is independent of age or sex. A person with BMI greater than 25 is considered overweight and a person with BMI greater than 30 is considered obese. In the U.S., the prevalence of overweight adults (25 years old and older) is estimated to be 59.4% for men, 50.7% for women and 54.9% overall (1). Recent data also suggest that approximately 10% of children are obese (2). Given the above statistics, obesity is clearly a major public health problem.

One of the major causes of the current epidemic of obesity may be the current environment characterized by increased availability of high fat and high carbohydrate food along with decreased physical activity (3,4). Epidemiological studies have shown that high levels of activity are associated with decreased BMI (5,6) and weight loss (7).

A number of recent studies have indicated that the physiological causes underlying the pathogenesis of obesity have a strong genetic component (8). In inbred populations, such as the Pima Indians and some Micronesian islanders, a high incidence of obesity and type II diabetes manifest (9). This suggests that these disorders may be inherited in an autosomal recessive manner (9). Further, a number of twin and population-based studies demonstrated that autosomal recessive genes control approximately 40% of the BMI variability (10,11). Interestingly, despite the apparent evidence for strong genetic contribution, no major locus controlling obesity has been identified in the human

Table 1. Body weight categories defined by body mass index (BMI).

Category	BMI (kg in body weight/ height in m^2)
Underweight	<20
Normal weight	20-24.9
Overweight	25-29.9
Obese	30-39.9
Severely obese	>40

population. The major reason may be due to: (a) genetic heterogeneity; (b) gene-gene interaction; and (c) gene-environment interaction.

Because of these difficulties, genetic influences on obesity have been explored in mouse models which share physiological and genetic similarities with humans. Consequently, utilizing mouse models to study genetics of obesity may provide opportunity to identify the genetic factors that cause obesity and/or identify common pathways that are important in regulation of energy balance in humans.

In the past seven years, several obesity genes have been identified (summarized in Table 1 in Part II). Among these genes, *agouti* was the first obesity gene to be cloned (12). Several dominant mutations at the *agouti* locus result in ectopic expression of the normal *agouti* gene under the control of a ubiquitous promoter. Ectopic expression of agouti causes a syndrome of obesity associated with yellow coat color, increased linear growth, hyperinsulinemia and type II diabetes (13). Mice carrying mutations at the agouti locus exhibit maturity-onset obesity (14). This makes the agouti mouse a good model for the common forms of human maturity-onset obesity associated with type II diabetes. In contrast, two other recently cloned obesity genes, *ob* and *db*, are associated with juvenile-onset obesity (obesity and diabetes respetively), occur as early as three to four weeks of age) (15). In addition, both *ob/ob* and *db/db* mice are hypercorticosteronemic while yellow agouti mice and humans do not have elevated levels of glucocorticoids (16). Therefore, identification of physiological pathways and factors involved in agouti-mediated obesity in a mouse model may provide information more relevant to human obesity and developing therapeutic drugs to treat human obesity.

II. RESEARCH OBJECTIVES

Currently, the precise molecular modes whereby mutations in the *agouti* locus and its ectopic expression contribute to the development of obesity are not fully understood. However, recent reports indicated that agouti may act as an antagonist to the hypothalamic receptor MC4-R (melanocortin receptor type 4), leading to hyperphagia and obesity (17). In addition, studies from our lab indicate that agouti induces a key de novo lipogenic enzyme, fatty acid synthase (FAS), and increases intracellular triacylglycerol (TAG) levels via a calcium-dependent mechanism (18). Since insulin has been shown to directly increase FAS transcription (19,20), it is possible that insulin and agouti interact to coordinately regulate FAS expression. Moreover, mice carrying agouti mutation (A^{vy}) and transgenic mice which ectopically express agouti under the control of β-actin promoter, overexpress FAS and leptin (21).

Consequently, the objectives of these studies are to determine: (1) whether agouti expression in adipose tissue or agouti treatment of adipocytes induces leptin and/or FAS expression, (2) whether agouti induction of FAS activity is due to increased FAS gene transcription, and (3) whether agouti response element(s), in the FAS promoter is distinct from the insulin response element.

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PART 2

LITERATURE REVIEW

I. RODENT SINGLE GENE MUTATIONS AND OBESITY

Several rodent single gene mutations that confer an obese phenotype have been described; *ob* (obese), *db* (diabetes), *fat* (fat), *tub* (tubby), *A* (agouti), and *fa* (fatty) (1) (Table 1). Among these obesity genes, *ob, db* and A^y genes are the most extensively studied.

Mice bearing mutations in *ob* or *db* gene exhibit a three-fold increase in body weight and a fivefold increase in fat content (2). *ob* and *db* locus mapped to different chromosomes in the mouse; *ob* on mouse chromosome 6 and *db* on chromosome 4. The wild type *ob* gene, which encodes a 167-amino acid protein named leptin, is primarily secreted by adipocytes and acts as afferent signal in a feedback loop regulating the size of adipose tissue mass (3). The *db* mutation results in indistinguishable metabolic and behavioral phenotypes as *ob*: type II diabetes, hyperphagia and decreased energy expenditure compared to lean controls (4). Genetic mapping studies indicate that *fa* and *diabetes* (*db*) are homologous loci coding for the leptin receptor, in the rat and mouse respectively (4). The *ob/ob* and *db/db* mice phenotypes are similar to that seen in animals with lesions in the ventromedial hypothalamus, suggesting that these mutations interfere with regulation of food intake in the central nervous system (1).

Tubby, an autosomal recessive mutation, mapping to mouse chromosome 7, is due to the mutation produced from a splicing defect in a novel gene with unknown function (5). The tubby mice develop a much milder form of obesity compared to the other rodent single mutations (6). Despite the fact that the function of this gene is undefined, a possible role may involve body weight regulation; as the normal transcript appears to be abundantly

Table 1. Rodent obesity mutations.

Mutations (type)	Gene	Rodent Chromosomes	Human Chromosomes	effects of mutations
A (dominant)	Agouti	2	20	ectopic overexpression
ob (recessive)	leptin	6	7	deficiency of leptin
db (recessive)	leptin receptor	4	1	defect in leptin signal
fa^{rat} (recessive)	leptin receptor	5	1	defect in leptin signal
fat (recessive)	carboxy peptidase E	8	4	processing of prehormone
tub (recessive)	tubby	7	11	undefined

expressed in the hypothalamus, a region of the brain involved in food intake regulations (7). It has also been postulated that the lack of normal tubby gene product causes neural degeneration, as mice carrying mutant tubby have retinal ganglion cell degeneration (7). Accordingly, apoptosis of neuronal cells in the feeding center of the hypothalamus may be responsible for the obese phenotype observed in tubby mutants (8).

The *fa* or *fatty* gene in rats has been mapped to chromosome 5, and mutations in this locus result in hyperphagia, hyperinsulinemia, hypertriglyceridemia, hypertrophy of adipocytes and insulin resistance (9). *fa* mutations in rats result in the leptin receptor lacking the extracelluar domain due to glutamine $\Rightarrow$ proline substitution (10).

The *fat* or carboxypeptidase E (CPE) mutation in mice results in moderate obesity, starting as early as 8 weeks of age (11). The normal function of CPE is to excise paired dibasic residues in carboxyl terminus of prohormones such as proinsulin (11). In the absence of normal CPE gene, levels of proinsulin are elevated (12). However, mice carrying mutant CPE are still viable due to compensatory processing of prohormones by residual the carboxypeptidase (13). The precise molecular mechanism by which CPE mutation results in obese phenotype has not been determined; as transgenic mice in which replacement of the normal CPE gene by pancreatic mutant CPE gene does not alter obese phenotype (13). Therefore, it is plausible that CPE may be involved in processing of other genes that are involved in body weight regulation, such as pro-opiomelanocortin (and therefore melanocyte stimulating hormone, MSH), causing derangement in normal inhibition of appetite stimulation via neuropeptide Y (NPY).

II. MOUSE AGOUTI GENE AND THE ROLE OF DOMINANT AGOUTI-LOCUS MUTATIONS IN OBESITY

A. Molecular Characteristics of the agouti gene

In wild-type mice, agouti refers to a pigmentation pattern (black hair with a subapical band of yellow). The *agouti* gene, in mouse chromosome 2, encodes a 131 amino acid paracrine factor. *agouti* gene is normally expressed only in the hair follicle during the hair growth period. Agouti protein causes hair pigmentation patterns by antagonizing binding of α-melanocyte stimulating hormone (α-MSH) to the melanocortin receptor type 1 (MC1-R). This results in suppression of cAMP production and subsequent shift from black (eumelanin) to yellow (phaeomelanin) pigment synthesis (14).

This regulated expression of *agouti* is disrupted in mice carrying dominant mutation (Table 2). Dominant mutant *agouti* locus contains at least 34 alleles that are arranged in a phenotypic dominance hierarchy with respect to one another (15-17). The most dominant alleles are viable yellow (A^{vy}) and lethal yellow (A^{y}) mutation, which confers a characteristic black apical band, subapical yellow band and black base with overall phenotypically yellow coat color (16). Another dominant allele, A^{iapy}, contains a viral promoter (intracisternal A particle or IAP) insertion between two of the non-coding exons preceding *agouti* start codon (17). This results in expression of agouti under the control of a viral promoter creating different phenotypes; yellow, intermediate or mottled, and pseudoagouti or black phenotypes (identical to wild-type phenotype). A^{iy} is another dominant mutation, which also rose from a IAP insertion into sequences upstream of the

Table 2. Naturally occurring mutant agouti and coat color patterns.

types of allele	coat colors	obesity phenotypes
A^{vy}	yellow	obese
A^{y}	yellow	obese
A^{iy}	variable (yellow, mottled or intermediate to pseudoagouti or dark)	variable
A^{iapy}	variable (yellow, mottled or intermediate to pseudoagouti or dark)	variable

first coding exon (18). Embryos that are homozygous for A^y allele die prior to implantation in the uterus, whereas viable yellow (A^{vy}) homozygotes are viable (19). This embryonic lethality is caused by complete inactivation of the neighboring ubiquitous gene Raly (reviewed in ectopic expression of agouti). Both animals carrying A^{vy} and A^y alleles display obesity phenotypes (20). The agouti gene is comprised of nine exons (A, B, C, D, E, 1, 2, 3 & 4). Exons A, B, C, D, E and exon 1 are non-coding exons and the last three exons (2, 3 and 4) encode the open reading frame (21) (Fig. 1). The processing of the agouti mRNA primary transcript results in two major types of alternatively spliced transcripts. These transcripts contain different lengths of non-coding first exons, which then lead to differential hair color expression. Form I contains 5' untranslated exons resulting in specific expression of yellow color hair in the dorsum and ventrum (18, 21). Form II transcripts arise from the same 0.8-kb mRNA, with agouti expression in ventral skin only (18, 21). Both forms I and II agouti transcripts have the same protein coding potential (20).

The agouti gene that is transcribed into an 0.8- KB mRNA is normally expressed in melanocytes within the follicular environment and function to control hair pigmentation (22). The functional Agouti protein contains a consensus signal peptide at the amino terminus followed by a highly basic region in the middle and a cysteine-rich carboxyl terminus. (23) (Fig. 2). The carboxyl domain is as biologically active in vitro as the full-length protein (24). Cysteine residues in this region all form disulfide bonds and are spaced similarly to the conserved ordering of cysteines in a large group of neurotoxins found in the venom of the primitive hunting spiders and cone snails (23).

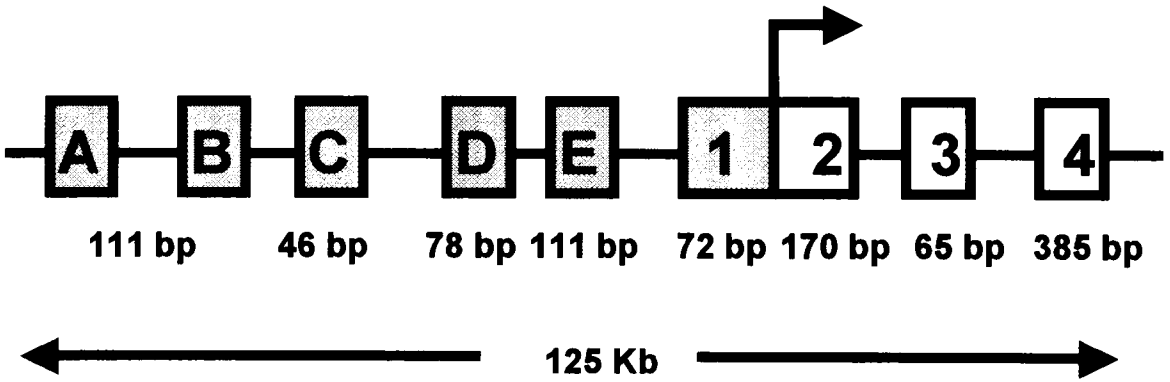

Figure 1. Genomic structure of *agouti* locus. Exons A, B, C, D, E, and 1 are non-coding exons. The location of the start codon is indicated with an arrow pointing toward exon 2, 3 and 4.

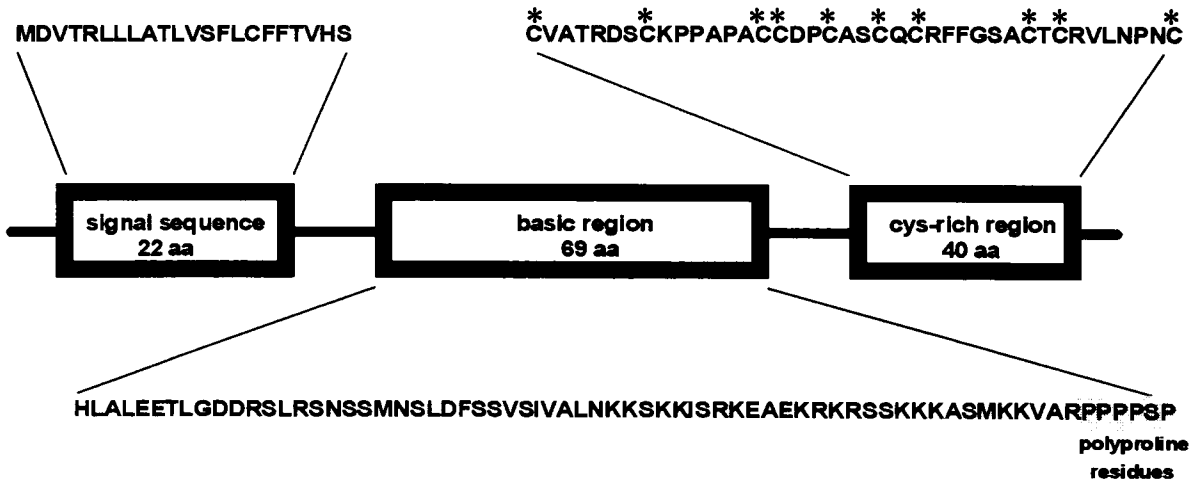

Figure 2. Structure of the Agouti protein. * Indicates cysteine residues that form disulfide bonds.

Transgenic mice with a mutation in the signal peptide of Agouti do not become yellow or develop obesity (23). Deletion of half of the central basic region of agouti significantly impairs the development of yellow fur or obesity (23). Mutations in the carboxyl terminus completely eliminated the potential for both yellow pigmentation and obesity, and decreased agouti inhibition of α-MSH binding to the melanocortin receptor and obesity (23).

The size of mRNA produced from the mouse carrying mutant agouti is about 1.1 kb, which is significantly larger than that of the wild type sized 0.8-kb mRNA (21). This mutation is due to a 5'-upstream 170-kb deletion that removes all but the first non-coding exon of ribonucleoprotein associated with lethal yellow (Raly) (Fig. 3). The Raly, expressed in a ubiquitous manner, maps 280-kb proximal to agouti on mouse chromosome 2 and encodes an RNA-binding protein (19). This deletion mutation results in transcription initiation from the ubiquitous promoter Raly to the second agouti exon (since this is the first coding exon). Transcription initiated from the Raly promoter still codes for the same Agouti protein as found in wild type mice, which becomes ubiquitously expressed.

B. Transgenic models of agouti expression/action: Role in body weight and coat color regulation

Utilization of transgenic models (Table 3) successfully demonstrated that ubiquitous expression of the normal *agouti* gene is responsible for the obese phenotype; when the wild-type *agouti* cDNA is placed under the transcriptional regulation of a

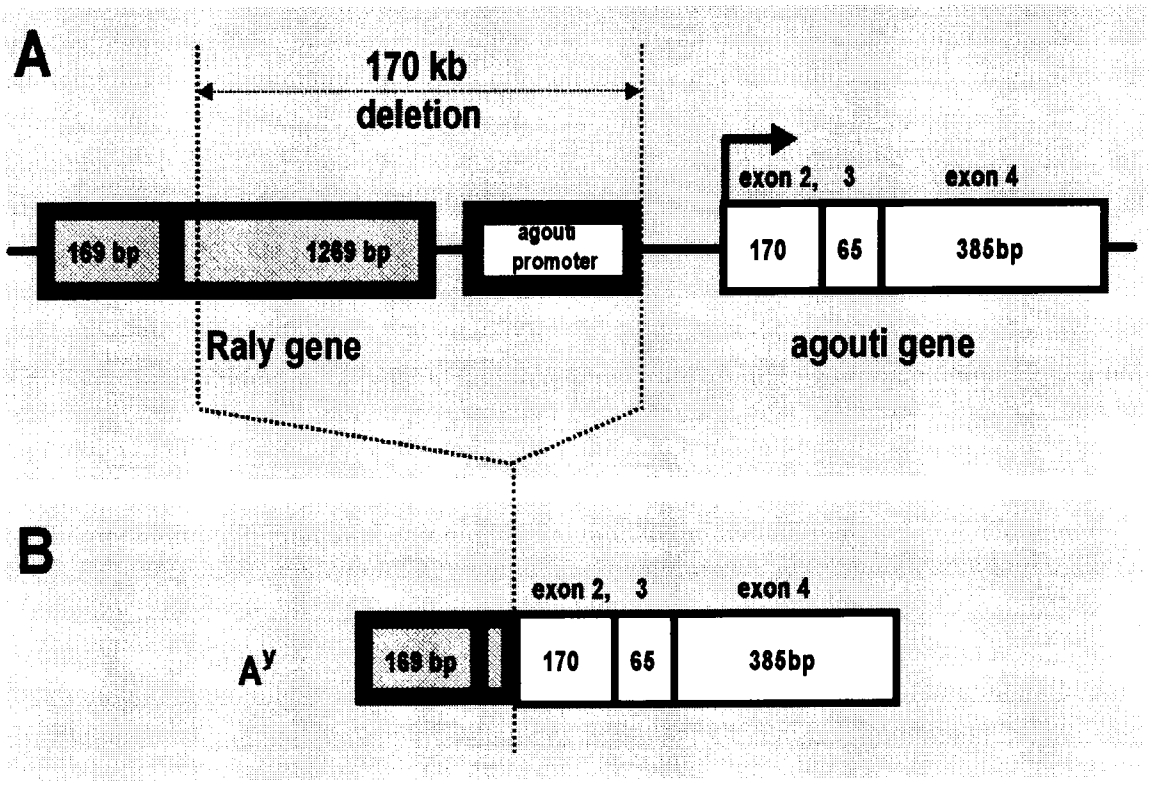

Figure 3. Proposed model for Raly/A^y gene. 3A. The 170 Kb deletion removes agouti promoter and most of the Raly gene except the first non-coding exon. 3B. The 170 Kb deletion results in ectopic expression of the *agouti* gene under the control of the ubiquitous Raly promoter.

Table 3. Physiological phenotypes of dominant *agouti* mutants and transgenic mice.

Type of Transgenics*	A^y, A^{vy}	BAP & PGK	aP2	MC4-R KO	AGRP	K14
Phenotype	obese	obese	normal	obese	obese	normal
Coat color	yellow	yellow	non-yellow	black	black	yellow
Food intake	increase	increase	normal	increased	increase	normal
Thermogenesis	moderate decrease	moderate decrease	normal	N/D**	N/D**	N/D**
Plasma leptin	increase	increase	normal	increase	N/D**	N/D**
Plasma insulin	increase	increase	normal	increase	increase	N/D**
Plasma glucose	increase	increase	normal	increase	increase	increase
Lipogenesis	increase	increase	N/D**	N/D**	N/D**	N/D**

* A^y, A^{vy}; dominant agouti mutations, BAP; β-actin promoter, PGK; phosphoglycerate kinase 1 aP2; adipocyte specific fatty acid binding protein promoter, MC4-R KO; melanocortin receptor type 4-knocout, K14; keratinocyte specific promoter, and AGRP; agouti related protein.
**Not determined.

ubiquitous promoter, such as β-actin (BAP) or phosphoglycerate kinase 1 (PGK) (25). The transgenic mice not only express the *agouti* mRNA in multiple tissues, but also develop obesity, hyperinsulinemia, hyperglycemia and yellow coat color (25). Transgenic (BAP line) males became 30 to 40% heavier and females 60-70% heavier than non-transgenic littermates (25). Furthermore, the basal core temperature was significantly depressed in transgenic compared to control mice (25), indicating that decreased thermogenesis may contribute to positive energy balance in these mice.

Agouti antagonizes the melanocortin receptor type 4 (MC4-R) that is expressed in regions of the hypothalamus involved in body weight regulation (26). Deletion of MC4-R recapitulated the yellow mouse syndrome including characteristic hyperphagia (27). This strongly suggests a possible involvement of agouti and MC4-R in regulation of food intake.

Agouti related protein (AGRP), a 132 amino acid protein that is 25% identical to agouti, is normally expressed in the hypothalamus and in adrenal glands (28). Like Agouti, AGRP contains a putative signal sequence and a cysteine rich carboxyl terminus, but lacks the region of basic residues and polyproline residues found in the middle of the agouti protein. Ubiquitous expression of AGRP also causes obesity without altering pigmentation (28). The severity of obesity syndrome in AGRP transgenic mice resembles that of the MC4-R knockout (28). In addition, AGRP levels are higher in obese mouse models such as *ob/ob* and *db/db* mice (28).

Transgenic mice expressing agouti under the control of the adipocyte specific fatty acid binding protein promoter, aP2, express very high levels of agouti in brown and white

adipose tissue (29). These mice exhibit normal insulin levels and do not become obese (29). This suggests that agouti expression in adipose tissue alone is not sufficient to bring about the obese phenotype. However, daily subcutaneous injections of insulin resulted in significantly body weight gain compared to insulin-treated non-transgenic littermates (29), suggesting that insulin is necessary for agouti to promote weight gain in vivo.

C. Physiological characteristics of yellow obese mouse

1. Food intake and body weight gain

Mice carrying agouti mutation exhibit increased body weight compared to their littermate control mice as early as 4 weeks of age (30,31). At three months of age, yellow mice show approximately twofold increase in body weight compared to control mice (32). The obese yellow mice are moderately hyperphagic and eat 10-36% more than the lean littermates (32, 33). However, decreased thermogenesis (whole body heat production) does not appear to be the major contributor in yellow mouse obesity; the thermogenic responses to a potent beta-agonist LY104119 were not different in obese yellow mice and normal a/a mice (34). Efficiency of food utilization (mean body weight gain divided by the number of calories consumed) was threefold higher in obese yellow mice compared to lean littermates (32). Taken together, these findings suggest that a major determinant of yellow mice obesity is the enhanced efficiency in storing consumed calories rather than hyperphagia or thermogenesis.

2. Lipid metabolism

The degree of obesity can be increased by feeding the yellow mice high-fat diets (32). Obese yellow mice have elevated blood lipids and ketones; fasting decreases blood ketones in obese yellow mice but increases them in normal mice (35). Adipose tissue transplantation between obese yellow mice and normal littermate controls have shown that the transplanted adipocytes increase or decrease in size depending on the genotype of the host, and not the genotype of the transplanted adipocytes (36). This suggests that the metabolic environment of obese yellow mice, not the intrinsic defect in adipocytes may be responsible for their obesity.

At 7 month of age, the lipogenic rate of obese yellow mice is six times higher than the littermate control mice (33) and have 50 % depressed basal lipolytic rate compared to control littermates (34). Addition of recombinant Agouti protein to human adipocytes inhibits basal and agonist stimulated lipolysis (37). Increased intracellular calcium, $[Ca^{2+}]_i$, also inhibits basal and agonist stimulated lipolysis in a dose-dependent manner (38). Moreover, the agouti inhibition of basal lipolysis is attenuated by the Ca^{2+} channel blockade nitrendipine (39). These data suggest that Ca^{2+} mediates antilipolytic effects of agouti.

Ca^{2+} has been implicated in insulin resistance and obesity (39). Intracellular calcium levels are elevated in obese yellow mouse compared to lean littermates (40). Treatment of adipocytes and pancreatic β-cells with recombinant agouti protein elevates $[Ca^{2+}]_i$ levels (40, 41). Agouti directly enhances FAS expression and triglyceride storage in 3T3-L1 adipocytes through a $[Ca^{2+}]_i$-mediated mechanism (43,44). Agouti-stimulation of

FAS expression in vivo and in vitro was inhibited by Ca^{2+} influx blockers (43, 44). These findings suggest that perturbations in calcium signaling by agouti may contribute to obesity of the yellow mouse.

3. Endocrine abnormalities

At six month of age, circulating insulin levels of the yellow mouse are approximately 20- fold higher than control lean mice (45). The elevated insulin levels in yellow mice (in the 21-day-old mice) are due to increased number of pancreatic beta cells (46). The mean number of beta cells/pancreas was significantly greater in the yellow mice than in the agouti mice while insulin content and body weight were the same (46). *agouti* gene is expressed in rat and hamster pancreatic cell lines and in human pancreas where agouti acts to increase insulin secretion (41).

Obese yellow mouse exhibit moderately increased glucocorticoid levels (47) which may contribute to obesity. Adrenalectomy decreased body weight, food intake, blood glucose, and insulin levels (48). However, adrenalectomy does not completely prevent the obesity in yellow mice (49). Corticosterone replacement in adrenalectomized yellow mice produced a dose-dependent increase in body weight that was associated with an increase in adipose tissue weight (48).

Growth hormone levels in genetically obese mice (*ob/ob*) (50) and *fa/fa* rats (51) are lower than their lean controls. In addition, a significant association between stunting and overweight has been demonstrated in human population (52). However, yellow obese mice exhibit approximately 10% increase in linear growth (53). The possibility that agouti

may mimic the effects of growth hormone has been excluded since growth hormone deficient yellow mice showed stunted growth and obese phenotype (54).

Pituitary gland is not required for the manifestation of yellow mouse obesity. Hypophysectomy decreased hyperinsulinemia in obese yellow mice; however, excess fat deposition was not prevented (55). Hypophysectomized yellow obese mice gained more weight than hypophysectomized control mice (56). These observations suggest that the pituitary endocrine axis is not involved in the expression of yellow mice obesity.

D. Mechanisms of agouti signaling: Role of melanocortin receptors

Agouti protein acts as an antagonist of melanocortin action of several recently cloned rodent and human melanocortin receptors (Table 4). These receptors belong to the G protein coupled seven transmembrane receptor family and are expressed in various tissues: MC1-R (α-MSH receptor) is expressed primarily in melanocytes and is involved in regulation of hair pigmentation (56), MC2-R (ACTH receptor) is primarily expressed in the adrenal cortex (57), MC3-R is expressed in neuronal cells in the brain as well as in the placenta and in the gut (57), MC4-R is primarily expressed in the brain (26, 57), and MC5-R is expressed in all tissues (26, 57).

Agouti protein, normally only found in the skin, is a high-affinity antagonist of the melanocyte-stimulating hormone receptor (MC1-R). Upon binding to MC1-R, Agouti blocks alpha-MSH stimulation of adenylyl cyclase, the effector through which alpha-MSH induces eumelanin synthesis. Involvement of the MC1-R in the development of obesity has been tested in the transgenic mice which overexpress the agouti gene in the skin (under the

Table 4. Melanocortin receptor subtypes.

Types	Tissue localization (human)	Tissue localization (mouse)	Ligands	Biological effects
MC1-R	pituitary, testis, lung, ovary, placenta, spleen, uterus	melanocytes macrophages	MSH, ACTH	pigmentation, immune function
MC2-R	adrenal, testis	adrenal adipocytes	ACTH	steroidogenesis Lipolysis
MC3-R	heart, testis GI tracts, brain	placenta, pancreas	MSH, ACTH	thermogenesis leptin regulation,
MC4-R	pituitary	brain, muscle	MSH, ACTH	thermogenesis, leptin-NPY food Intake regulations
MC5-R	all tissues	all tissues	MSH, ACTH	thermogenesis immunomodulation, Sexual behavior

control of skin specific promoter, keratin-14). K-14 mice exhibited yellow coat colors resembling dominant agouti allele phenotypes; however, these mice did not become obese (58). In addition, mice lacking MC1-R display black coat color and obesity (59). These data suggest that antagonism of Agouti to MC1-R, and subsequent alteration of hair pigmentation is not involved in yellow mouse obesity.

Agouti protein does not circulate in blood as demonstrated in parabiosis experiments (60). Thus, effects of agouti in the development of obesity must occur within local tissues that expresse agouti. Hypothalamus plays a fundamental role in the control of food intake. Tow of the melanocortin receptors (MC3-R and MC4-R) have been found in this region of the brain. Agouti acts as a potent antagonist to hypothalamic MC4-R receptors (61). In addition, MC4-R knockout mice resulted in increased food intake and marked obesity (27). Administration of a MC4-R agonist (MTII) inhibited feeding and this inhibition was blocked when MC4-R antagonist (SHU9119) was co-administrated (27). Consequently, the obesity caused by ectopic expression of agouti in the obese yellow mice may involve antagonistic effects of agouti to these melanocortin receptors in the hypothalamus.

Agouti related protein (AGRP) is a novel gene product which shares 25% homology with Agouti protein and has been implicated in the control of feeding behavior. AGRP is primarily expressed in the hypothalamus and adrenals (28). AGRP shares the highest degree of homology with Agouti protein in the carboxyl terminus region that is critical for binding to their receptors (62) and AGRP exerts comparable effects with agouti in vivo. Like β-actin driven agouti expression, β-actin driven AGRP expression caused a

similar obesity phenotype (28). This effect of AGRP is suggested to be mediated through MC3-R and/or MC4-R as AGRP is a very selective antagonist to MC3-R and MC4-R (28). In addition, a C-terminal fragment of AGRP, when administered intracerebroventricularly (ICV) into rats, increased food intake (63). Furthermore, agouti related protein is upregulated in *ob/ob* and *db/db* mice (28) providing further evidence for its involvement in the development of obesity.

Collectively, these recent findings strongly suggest that agouti and other related proteins regulate food intake centrally by antagonizing melanocortin receptors.

E. Mahogany and mahoganoid

The mouse mutations mahogany (*mg*) and mahoganoid (*md*) are negative modifiers of the agouti coat color gene (64). Animals mutant for *md* or *mg* synthesize very little or no phaeomelanin (64) and *md* and *mg* suppress the effects of A^y on both coat color and obese phenotype (64). The MC1-R mutations suppress the effects of *md* and *mg*, but do not affect plasma levels of α-MSH and of ACTH suggesting that *md* and *mg* interfere directly with Agouti signaling (64).

F. Human homologue of agouti and melanocortin receptors

The human homologue of the *agouti* gene, referred to as agouti signaling protein (ASIP), maps to human chromosome 20 encodes a 132 amino acid protein, which is 85% identical to the mouse gene. Human agouti is expressed in adipose tissue (65), testis,

ovary, heart, liver, kidney and foreskin (66). Overexpression of ASIP in transgenic mice produces a yellow coat, and its expression in mouse melanoma cells blocks the α-MSH-stimulated accumulation of cAMP. Interestingly, ASIP on chromosome 20 maps near the locus for maturity-onset diabetes of the young (MODY1) (66). Whether mutations of human agouti are responsible for MODY1 has not been determined.

Human adipocytes express MC1-R, MC2-R, MC4-R and MC5-R, and ASIP has been shown to be a potent antagonist for α-MSH binding to MC1-R. Whether ASIP is normally expressed in the human brain or exert antagonistic effects on brain specific melanocortin receptors are currently unknown. A recent study showed absence of linkage association between ASIP loci and obesity phenotypes. Nevertheless, linkage association of MC4-R and MC5-R and obesity related phenotypes have been suggested using restriction fragment-length polymorphism (RFLP) (67).

III. METABOLIC PATHWAYS AND OBESITY

A. Energy metabolism and obesity

Obesity is a multifactorial disease caused by the influence of both genetic and environmental factors. A long-term excess energy intake (above energy expenditure) creates chronic positive energy balance and causes weigh gain. Several reports showed a positive correlation between body fat content (or adiposity) and dietary intakes in epidemiological studies (68,69,70). Furthermore, propensity to gain weight is greater at low levels of physical activity (71).

Interestingly, obesity in humans may be an evolutionary disease as more efficient metabolism was necessary for survival of primitive culture characterized by regular episodes of fasting and intense physical activity (72). In contrast, modern cultures are characterized by overeating and sedentary life-style. Therefore, interplay between environmental factors and genetic factors may be an important part of the mechanisms allowing humans to adapt to their environment.

A number of environmental factors that are linked to obesity such as nutrients may also control activity of metabolic genes that influence obesity. For example, increased insulin secrection due to chronic increased intake of dietary carbohydrate increases expression of lipogenic genes (73) and this may contribute to obesity.

B. Nutritional and hormonal regulation

Obesity is generally associated with increased fat cell size (74). This hypertrophy

of adipocyte is due to coordinate activation of several enzymes involved in triglyceride storage including FAS, lipoprotein lipase (LPL), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and malic enzyme (75).

Several dietary factors modulate lipogenesis. High carbohydrate diet increases lipogenesis (76) while high-fat diet impairs glucose metabolism in muscle by reducing transcription of glucose transporter type 4 (glut4) (77).

Hormonal induction of lipogenesis may also contribute to obesity. Growth hormone exerts an acute insulin-like effect by increasing lipogenesis and repressing lipolysis via activation of phosphodiesterase, leading to reduction of cAMP levels (78). Angiotensin II, recently shown to be synthesized and secreted from adipocytes (79), also controls lipogenesis by inducing FAS and glycerol-3-phophate dehydrogenase activities in adipocytes (79). Glucocorticoids also induce de novo lipogenesis (80,81). Glucagon-like peptide type 1 (GLP-1) exerts insulin-like effect on glucose metabolism in adipocytes, hepatocytes and skelatal muscle cells (82).

Elevated insulin levels are associated with the development of obesity along with hyperphagia, and adrenal hyperfunction (83). Insulin also directly regulates adiposity by enhancing lipogenesis and by decreasing lipolysis (84-86).

The lipogenic effect of insulin is clearly defined in several studies. Chronic administration of exogenous insulin resulted in approximately 11-fold increase in de novo biosynthesis of palmitate in rats (87). Genetically obese animals have hypernsulinemia and corresponding increase in de novo lipogenesis levels (43,88, 89). This effect of insulin is due to increased glucose uptake by glucose transporter type 4 (glut4) (90).

IV. REGULATION OF LIPOGENIC GENE EXPRESSION

A. Fatty acid synthase

Adiposity or adipose cell volume reflects the balance of lipolysis and lipogenesis. Insulin induces lipogenesis by stimulating glucose conversion into triacylglycerols (TAGs) in adipocytes (91). This lipogenic effects of insulin leading to storage of TAG correlates with increases in de novo lipogenic enzymes, acetyl CoA carboxylase (ACC) and fatty acid synthase (FAS). ACC catalyzes the rate-limiting step in fatty acid biosynthesis. However, ACC is subject to short-term regulation while FAS is associated with long-term regulation of fatty acid synthesis in response to nutritional and hormonal changes.

FAS maps to mouse chromosome 11 and human chromosome 17 (92,93, 96). FAS is a key de novo lipogenic enzyme which catalyzes the synthesis of palmitate from acetyl-CoA and malonyl-CoA in the presence of NADPH. The FAS gene has been cloned in various other species, including rat (94), chicken (95), and mouse (96). FAS protein consists of seven subunits with specific functional domains; ketoacyl synthase, malonyl-acetyl transferase, dehydrase, enoylreductase, ketoreductase, acyl carrier protein and thioesterase juxtaposing head-to-tail forming two distinct moieties for fatty acid assembly (97). Production of palmitic acid (16:0) by FAS occurs through a cyclic process in which malonyl moiety undergoes series of condensation/decarboxylation reactions resulting in elongation of acyl group by two carbons per cycle yielding fully saturated sixteen carbon fatty acid as a final product. The entire process takes seven cycles of elongation and uses fourteen molecules of NADPH (97).

Activity of FAS is highly regulated by hormonal and nutritional factors; it is increased by feeding (98), thyroid hormone (99) and insulin (99), and decreased by starvation (98), cAMP (98), and polyunsaturated fatty acids (100). The FAS gene is primarily regulated at the transcriptional level (101,102). However, posttranscriptional regulation of FAS mRNA also has been described (99,103). In addition, FAS levels are elevated in obese animals (88,104,105). Liver and adipose tissue are major tissues in which de novo lipogenesis occur. However, adipocytes recently have been suggested to play a major role (relative to liver) in human fed carbohydrate rich diets (106).

B. Insulin regulation of FAS gene transcription

Insulin-stimulated FAS expression is due to increased transcription of FAS gene (107, 109, 110). This transcriptional regulation is mediated by regulatory sequences in the proximal promoter region of the FAS gene (107). Mutational analysis of this region showed that sequences between -68 and -60 are essential for recognition and interaction with trans-acting factor(s) (107). These sequences retain their insulin responsiveness when ligated to a heterologous promoter and transfected to 3T3-L1 adipocytes. These studies demonstrated that the insulin response sequences of the FAS gene are located in the region from -67 to –52 (107). These same sequences mediated insulin like growth factor-1 (IGF-1) regulation of FAS in undifferentiated preadipocytes (108).

Among the trans-acting factors that up-regulate FAS gene transcription through the insulin responsive sequences (IRS) are the upstream stimulatory factors 1 and 2 (USF1 and USF2) (109). The -65/-60 E-box motif (5'-CATGTG-3') is required for insulin

regulation and USFs are in vivo components of the insulin response complex. In addition, mutation of the -65/-60 E-box sequence abolished the insulin response in both transiently and stably transfected 3T3-L1 adipocytes (109). Further, cotransfection of USF1 and USF2 expression vectors with the FAS promoter-luciferase reporter constructs increased insulin-stimulated FAS promoter activity (109). Cotransfection of dominant negative USF1 and USF2 mutants lacking the DNA binding domain inhibited the insulin stimulation of the FAS promoter activity (109).

Adipocyte determination differentiation dependent factor (ADD-1 or sterol regulatory element binding protein, SREBP-1) is another type of transcription factor which mediates its effect on adipocyte energy homeostasis through the E-box motif (at -64 to –59, previously identified as IRS) in the FAS promoter (110). Results from a recent study demonstrated that insulin induced expression of FAS in cultured adipocytes via E-box sequences (110). Thus USF1/USF2 and ADD-1/SREBP-1 mediate insulin responsiveness via the E-box motif in the FAS promoter.

Additional insulin responsive sequences have been identified; these sequences are located at DNase I-hypersensitive sites (-518 to -495) with structure, 5'-GCCT, 6-bp spacer and a 3'-palindrome (111,112). The identity of transcription factors that bind to this region is currently unknown.

Four different regions within -568 to -468 of FAS promoter bind specific nuclear factors; A box (-564 to -536), B box (-534 to -506), FAS Insulin- Responsive Element 1 (FIRE1) (-514 to -498) and C box (-489 to -470). Sp1 binds to A and C boxes while B box binds to nuclear factor 1 (NF-1) and FIRE binds to nuclear factor Y (NF-Y) (113). C

box and FIRE are not directly involved in insulin regulation, as no difference in response to insulin was detected. NF-Y is a transcription factor with more specific function such as energy metabolism; several genes involved in gluconeogenesis and fatty acid metabolism are regulated by NF-Y. NF-1 binding elements (A boxes) contain 12 out of 13 bases of nucleotide bases that are contained in enhancer region of hepatitis B virus, polyomarvirus, a major histocompatibility complex class II antigen promoter (114). This suggests that A box may bind other factors than NF-1. NF-Y and Sp1 binding sites are in close proximity. NF-F and Sp1 bind DNA cooperatively (NF-Y*Sp1*DNA) rather than individually (NF-Y* DNA and Sp1*DNA) (113). Sp1 is a general transcription factor which is involved in regulation of many housekeeping genes, either alone or by acting synergistically with other transcription factors (115). Further, Sp1 and IRE are in juxtaposition in several genes such as GAPDH (116) and FAS (107). Moreover, synergistic interaction between Sp1 and SREBP-1 has been found in the low density lipoprotein promoter region (117).

Consequently, transcriptional induction of lipogenic genes such as FAS may involve NF-1 and Sp1 interaction (cooperate binding to DNA), which creates distortion in the double helix, allowing its opening for transcription factor binding (118). This induction of FAS gene expression may then result in increased transcription of the FAS gene and subsequently adiposity.

C. Polyunsaturated fatty acids (PUFA) regulation of FAS gene

Dietary polyunsaturated fatty acids (PUFA) induce hepatic peroxisomal fatty acid oxidation and suppress lipogenic gene expression (S14 and FAS) (119). Saturated and

monounsaturated fatty exert no suppressive action (119).

Recently, a group of fatty acid activated nuclear transcription factors termed peroxisome proliferator activated receptors (PPARs) were cloned (120). The peroxisome proliferator-activated receptors (PPARs) are members of the steroid/thyroid nuclear receptor superfamily of ligand-activated transcription factors. PPARs are expressed at high levels in the liver and adipocytes regulating lipid oxidation and adipocyte differentiation (120). Accordingly, PPAR has been implicated as a mediator of fatty acid effects on gene transcription. However, results from recent studies have shown that PPAR regulation of lipogenic gene transcription involve separate and independent mechanisms. Rats and mice fed a potent PPAR activator, 5,8,11,14-eicosatetraynoic acid (ETYA) exhibited increased peroxisomal acyl-CoA oxidase mRNA abundance, but PPAR activation neither suppressed fatty acid synthase transcription nor reduced the level of fatty acid synthase mRNA (100). Similarly, treating rat hepatocytes with arachidonic acid (20:4, n-6) suppressed FAS expression but had no effect on acyl-CoA oxidase (100). Thus, it appears that the PUFA regulation of gene transcription involves a PUFA-response factor that is independent from PPAR.

V. LEPTIN AND ENERGY BALANCE REGULATIONS

A. Introduction

The energy balance (the difference between energy intake and expenditure) is maintained by a feedback system. One of the key elements involved in the feedback regulation of energy stores is an adipocyte derived peptide hormone named leptin. Leptin is secrected primarily by adipose tissue and acts in the central nervous system (CNS) to decrease appetite and increase energy expenditure. The appetite suppressing effect of leptin is mediated by decreased levels of a potent appetite stimulator neuropeptide Y (NPY). Chronic administration of neuropeptide Y produces hyperphasia and obesity (121). Conversely, NPY-induced obesity results in increased circulating leptin levels (121). In addition to its effects on food intake, leptin increases energy expediture by inducing expression of uncoupling proteins (122). Leptin levels positively correlates with the amounts of body fat and its absence produces massive obesity. Leptin thus functions as the afferent component of a negative feedback mechanism with the CNS to control adipose tissue mass.

B. Leptin

The mutant gene responsible for obesity in the *ob/ob* mouse was identified by positional cloning in 1994 (123). Parabiosis studies (2), in which circulatory systems of two obese mice *ob/ob* and *db/db* were connected to lean wild type mice, resulted in weight loss of *ob/ob* mice and no weight change in *db/db* mice. This suggested that wild type

mice were physiologically capable of producing a circulating factor that controls body weight. This result also suggested that *ob/ob* mice lack the functional form of this factor while *db/db* may have defective signaling pathways. This circulating factor, leptin, was recently identified by positional cloning (123).

The primary product of the *ob* gene, leptin, is a 167-amino acid protein produced primarily in fat cells of several species studied, including humans. Leptin binds to one or more proteins in the circulation (124). Normally, soluble hormone receptors present in serum modulate the bioavailability and bioactivity of hormones. Several proteins in serum bind to leptin (124). Approximately, 10% of these putative leptin binding proteins are leptin receptors (124). The identity of other binding proteins remains to be determined.

The percentage of free leptin is higher in obese than in lean individuals (125). Indeed, plasma leptin levels are closely correlated with body fat both in humans and in rodents (48,124,125).

C. Leptin receptors

The leptin receptor gene, on mouse chromosome 4, encodes a protein consist of 894 amino acids. Leptin receptor shares high homology with the cytokine receptor family. The extracellular domain of this receptor is comprised of 816 amino acids, the transmambrane domain is consisted of 23 amino acids, and the cytoplasmic domain is comprised of 34 amino acids (126). Leptin receptors are expressed in lung, kidney, and brain (choroid plexus and hypothalamus) (126).

In humans and rodents, two major forms of leptin receptors are expressed; the

short form (OB-RS), lacks signaling capability, is detected in kidney, lungs, and choroid plexus (128). In contrast, majority of OB-R long form (OB-RL) is expressed in the hypothalamus and low levels of expression is present in peripheral tissues (128). The long form mediates the weight reducing effects of leptin (128). The OB-RS transports leptin across the blood brain barrier (128). *db/db* mice and *fa/fa* rats exhibit a mutation in the long form of the receptor (129,130). In *db/db* mice, mutations in OB-RL is due to G to T substitution which produces a new splice donor and subsequent introduction of a premature stop codon, resulting in receptors which lack the intracellular domain) (129). In *fa/fa* rats, glutamine to proline substitutions results in alteration of the extracellular domain of the receptor (130).

The long-form of leptin receptor transmits its information via the Janus Kinases (JAK), which phosphorylate transcription factors of the Signal Transducers and Activators of Transcription (STAT) family. Rat and human hepatoma cell lines transfected with human OB-RL display enhanced leptin binding and activation of STAT proteins (128). In addition, leptin stimulated recruitment of phosphatidylinositol 3-kinase to insulin receptor substrate-2 (128). However, tyrosine phosphorylation of insulin receptor substrate-2 does not modulate immediate cell response to insulin treament (128).

The long forms of leptin receptor are expressed at low levels in tissues other than the hypothalamus including liver, heart, skeletal muscles, endocrine pancreas (121). However, the role of leptin receptors in these tissues is currently not known.

D. Physiological actions of leptin

Daily injections of recombinant ob protein decrease body weight, percent body fat, food intake and serum concentrations of glucose and insulin (131). Leptin also increase the metabolic rate, body temperature, and activity levels of *ob/ob* mice (132). This weight reducing effects of leptin is possibly mediated through neuropeptide Y (NPY), a potent appetite stimulator expressed in the hypothalamic arcuate nucleus (ARC) that project to the paraventricular nuclei (PVN) and dorsomedial nuclei (DMH). Recombinant leptin injected into the lateral ventricle of fasted adult Wistar rats inhibited food intake by 20-25% between 2 and 6 h after administration (121). Leptin treatment significantly reduced NPY concentrations by 20-50% in the ARC, PVN, and DMH (121).

Intracerebroventricular NPY administration induced sustained hyperphagia and excessive weight gain (121). In NPY-treated rats, plasma leptin levels were significantly higher than control mice (121). Leptin decrease NPY levels (121). This surpressive action of leptin on NPY in the PVN mediates leptin's hypophagic and thermogenic actions (121). Thus, these two factors, NPY and leptin, may interact to regulate body fat mass and energy balance.

Despite the fact that *ob* and *db* mutations in mice result in severe obesity and diabetes, such mutations do not appear to be responsible for most human obesity. Since human *ob* gene cloning in 1994 (123), a few isolated cases of human *ob* gene mutation and leptin receptor mutations have been reported. A recent study of two severely obese children (with very low serum leptin levels despite of their markedly elevated fat mass) showed mutation in *ob* gene representing a sub-set of human populations with genetic

obesity resembles *ob/ob* mouse obesity. Administration of functional leptin protein to an obese human adult subject (who lacks functional leptin) and subsequent reduction in their fat mass has been demonstrated (Farooqi et al., special report presented in International Congress of Obesity meeting in Paris, France, 1998).

The most of human obese subjects show 72% higher levels of ob gene expression than their lean counterparts (124). This suggests that human obesity may be predominantly due to a defect in the leptin receptors (as in *db/db* mice model). Nevertheless, results from recent studies showed that leptin receptor gene are not a common cause of human obesity; mutations in the leptin receptor coding sequences are similar in both lean and obese humans (133).

In addition, the human ob mRNA level varied in adipose tissue from region to region (subcutaneous vs. omental adipose tissue) even in the same individual (134,135). Human ob mRNA is expressed in mature adipocytes but not in stromal vascular cells, fibroblast, or endothelial cells (135). This suggested that human *ob* gene expression is induced during cell maturation or differentiation.

E. Regulation of leptin production

Leptin production is regulated by several hormones; both leptin gene transcription and secretion are modulated by adrenal corticoids (136-139). In vivo as well as in vitro studies in rodents and human have shown that glucocorticoid enhance leptin gene transcription and circulating leptin levels (136-139). The precise molecular mechanisms wherby glucocorticoid induce leptin secretion is unknown. Catecholamines, via beta-

adrenergic receptors and cAMP (140), and thiazolidinediones via PPARγ, inhibit leptin expression (141). Insulin stimulation of leptin production has been demonstrated in cultured adipocytes and streptozotocin-diabetic animals treated with insulin (142, 143). In humans, leptin levels correlate with basal insulin levels (144, 145). *ob* gene transcription and plasma leptin levels are severely reduced by fasting (146) and increased after food intake (131).

G. Agouti-leptin interaction

Several neuropeptides including α-MSH are derived from the precursor pro-piomelanocortins (POMC) gene. α-MSH may act as an agonist at the MCR-4 to decrease feeding, possibly via interaction with the leptin signaling pathway. In the arcuate nucleus, 30% of the POMC neurons express the long form of leptin receptor and the arcuate POMC mRNA is upregulated by leptin (147). In addition, POMC levels were reduced in *db/db* and *ob/ob* compared to lean animals, while leptin injection to *ob/ob* mice but not *db/db* enhances POMC levels to those found in lean controls (147). Thus, it is conceivable that high levels of leptin induce POMC, which then leads to increased MC4-R activation via MSH, resulting in decreased food intake. Consistent with high leptin levels in obese yellow mice, transgenic mice ubiquitously expressing agouti also exhibited higher levels of leptin compared to non-transgenic controls. Despite these high levels of leptin, this "leptin resistance" is not due to genetic defects blocking leptin action in obese yellow mice. This conclusion was supported by recent studies by Boston et al., (148) which demonstrated that removal of leptin from the obese yellow mice (double mutant for Agouti and ob)

restores complete leptin sensitivity. Melanocortin ligands were able to regulate food intake in leptin deficient (*ob/ob*) mice (61), suggesting that MCR signaling is intact in these mice and does not require functional leptin to regulate feeding behavior. Selective antagonist of the MC4 receptor (HS014) administered ICV, significantly and in a dose-dependent manner increased food intake by 100% at 4 h after the injections (149). These studies suggest that agouti and leptin act independently to regulate food intake.

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PART 3

EXPERIMENTAL INVESTIGATIONS

I. REGULATION OF LEPTIN BY AGOUTI[1].

A. Abstract

Dominant mutations at the mouse agouti locus results in ectopic expression of the agouti gene, resulting in yellow coat color, type II diabetes, insulin resistance and obesity. We hypothesized that agouti directly induce adipocyte hyperptrophy and induce subsequent leptin secrection. Accordingly, we used transgenic mice expressing agouti ubiquitously (under the control of β-actin promoter, BAP20) or in adipocytes (under the control of aP2 promoter; aP212) to examine changes in leptin secretion levels. We have previously shown that insulin injection to aP2 transgenic mice leads to a significant weight gain. Our current studies demonstrated that plasma leptin levels are five to six-fold higher in BAP20-transgenic mice compared to littermates. Similarly, aP212 transgenic mice exhibited an approximate two-fold increase in plasma leptin compared to controls. Insulin treatment of aP2 mice increased intracellular leptin content without affecting plasma leptin levels. These findings were further confirmed by assaying leptin secretion into culture media from 3T3-L1 adipocytes treated with recombinant agouti protein (100 nM) and/or insulin (100 nM). Agouti treatment significantly increased leptin secretion into culture media, while insulin caused no significant effect compared to control indicating that insulin enhances leptin synthesis but not secretion. These results suggest that agouti-increased leptin synthesis and secretion may serve to limit agouti-induced obesity.

[1] This manuscript has been submitted for publication in similar form with authors Claycombe, K.J., Xue, B., Mynatt, R.L., Zemel, M.B., and Moustaïd-Moussa, N. in: Diabetes.

B. Introduction

Agouti, a paracrine factor composed of 131 amino acids, is normally secreted within hair follicles during the hair growth period (1). Secreted agouti protein regulates hair pigmentation by competitive antagonism of α-melanocyte stimulating hormone at its receptor (MC1-R), resulting in a switch from eumalanin to phaeomelanin production (2). Dominant mutations at the agouti locus result in ectopic expression of the agouti gene causing yellow coat color, marked obesity, hyperinsulinemia, type II diabetes and cancer (3,4). Although agouti was the first obesity gene to be cloned (5), its role in adipocyte metabolism and its effect on secreted adipocyte hormones, such as leptin, has not been determined.

Leptin, the *obese* gene product, is produced primarily in adipose cells (6). Leptin plays a key role in regulation of food intake and energy balance primarily by binding to hypothalamic leptin receptors (OB-R) (7). Changes in adipose tissue mass generally correlates with changes in plasma leptin levels (8,9) and plasma leptin is elevated in several obese animal models (including yellow agouti mice) and in humans (10).

A limited number of studies demonstrated agouti-leptin interaction in central nervous system (CNS). Leptin stimulates hypothalamic pro-opiomelanocortin (POMC) gene expression (11). POMC serves as a precursor to α-melanocyte-stimulating hormone (α-MSH) which is an endogenous ligand for melanocortin receptor 3 and 4 (MC3-R and MC4-R) in the hypothalamus (12). Agouti and agouti related protein (AGRP) antagonize melanocortin MC3-R and MC4-R (12).

In the periphery, leptin exerts direct effects on the metabolic function of adipose

tissue; leptin increased the rate of lipolysis and lipoprotein lipase mRNA (13).

To date, no studies addressed pheripheral actions of agouti on leptin secretion. We have previously shown that agouti regulates adipocyte metabolism (14,15). We also demonstrated that adipocyte specific expression of agouti exhibit a significant weight gain (16) and increased fat tissue mass (unpublished data). Whether expression of agouti in the adipose tissue and subsequent increase in adipose tissue mass leads to increase in leptin levels have not been determined. We hypothesized that agouti induces lipogenesis leading to increased leptin secretion to limit this agouti-induced adiposity via autocrine mechanisms. Accordingly, we investigated the role of adipocyte specific expression of agouti in regulating leptin levels in plasma and adipose tissue using transgenic mice that expresse agouti under the adipocyte specific promoter aP2. Our data demonstrated that expression of agouti in the adipose tissue results in increased plasma leptin levels. In addition, administration of insulin enhanced leptin synthesis but not secretion; in vitro studies performed in murine 3T3L-1 adipocytes further supported these differential in vivo effects of agouti and insulin on leptin secretion.

C. 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 (16, 17). Transgenic mice used in these studies were maintained in our breeding colony at the University of Tennessee, Department of Nutrition. All mice were weaned at 4 weeks of ageand 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 nontrangenic 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 (16), while agouti was expressed in all tissues from BAP20 mice (17).

Insulin injection: Twelve-week old male aP212 line mice were subcutaneously injected with Human insulin (Eli Lily) at a daily dose of 1 unit/day/mouse for two weeks. Insulin (100 units/ml) was diluted prior to daily injection with phosphate buffered saline (PBS) to make final injection volume of 200 :l (1 unit). The systemic effect of injected insulin was confirmed by measuring blood glucose levels. The blood glucose levels decreased significantly (immediately following subcutaneous the insulin injection) and normalized within 4 hours after the injection (data not shown) in both transgenic and in control mice. Prior to tissue collection, mice were anesthetized using pentobarbital and blood was obtained by cardiac punture. Animals were killed by removal of heart. Adipose tissues (epididymal, perirenal and 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.

Leptin measurements: Blood was collected via heart puncture using heparinized tubes. Plasma was collected and 100 ul was used in a radioimmuno assay (RIA) using a kit purchased from Linco (St Charles, MO) to determine leptin levels. Adipose tissue was homogenized in phosphate buffered saline (PBS) and 100 :l of the homogenized tissue

extract were used in the RIA to determine intracellular leptin levels, which were then corrected to protein content in the extracts; protein content was assayed using Bradford method (18).

Cell culture: 3T3-L1 cells were grown and differentiated as previously described (19). Briefly, cells were grown to confluency in standard Medium: Dulbecco's modified Eagle's (DMEM) media supplemented with 10% fetal bovine serum (FBS). Adipocyte differentiation was induced by treating confluent cells with dexamethasone (250 nM) and iso-butylmethylxanthine (0.5mM). Cells were then maintained for 3 additional days in standard medium then changed to serum-free medium (containing 1% BSA) followed by treatment with agouti protein and/or insulin as indicated in the figure legends.

Statistical analysis: Multiple and nested analysis of variance (SAS, Cary, N.C.) were used to compare the data. All data are expressed as mean +/- SE at significance level of 0.05.

D. 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 six fold higher plasma leptin levels compared to controls ($p<0.0005$) (Fig. 1). Transgenic mice expressing agouti only in adipose tissue (aP212) also exhibit a two-fold increase in plasma leptin levels compared to controls (Fig. 1, $p<0.05$). Similar plasma leptin levels were obtained in another aP2 transgenic line (data not shown) as

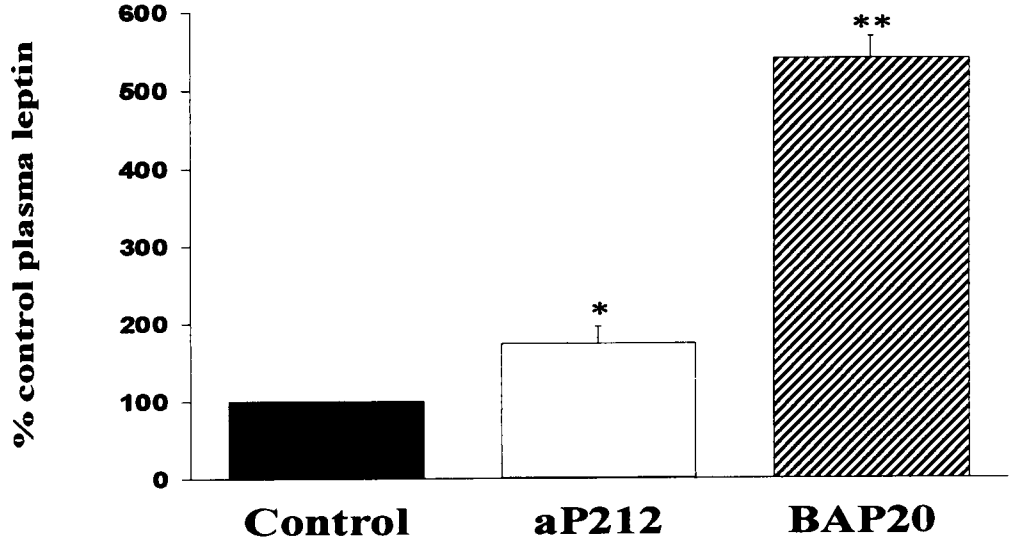

Figure 1. Effects of ubiquitous and adipose tissue expression of agouti on plasma leptin levels. 10–week old transgenic mice ubiquitously expressing agouti (BAP20, n=8) or specifically expressing agouti in adipose tissue (aP212, n=10) were used. The fold increase in plasma leptin levels over respective nontrangenic control littermate is shown.

demonstrated in aP212 mice. These data demonstrate that adipose specific and ubiquitous expression of agouti lead to increased circulating levels of leptin.

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).

In agreement with increased plasma leptin levels in transgenic compared to control mice, adipose tissue leptin levels were also significantly higher in transgenic vs. control mice (Fig. 3A). This indicates that agouti increases both leptin synthesis and secretion. Interestingly, while insulin treatment did not result in changes in plasma leptin levels, administration of insulin to aP212 transgenic mice resulted in a significant increase in adipose tissue leptin levels (1.61 ± 0.31 vs. 1.10 ± 0.01ng/ml, in insulin and saline respectively) (Fig. 3B). However, no significant effect of insulin was observed in control mice (data not shown). Taken together, the above results demonstrate that agouti increases both leptin synthesis and secretion while insulin increases leptin synthesis without modifying its secretion.

Differential effects of agouti and insulin on secreted and intracellular leptin were further examined using 3T3-L1 adipocytes. The results from RIA using culture media collected from 3T3-L1 adipocytes treated with agouti, insulin or a combination of agouti and insulin are shown in Figure 4. 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/m,

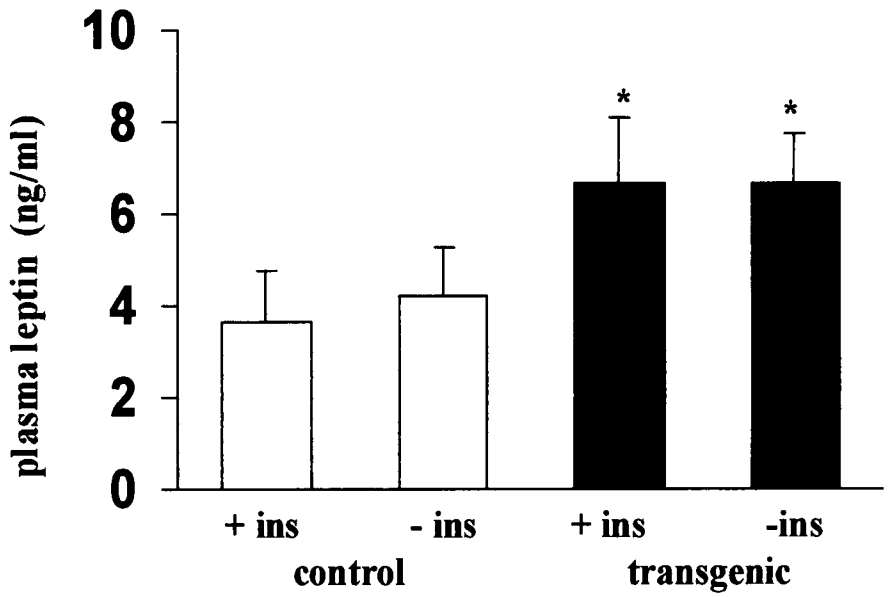

Figure 2. Effects of insulin administration and agouti expression on plasma leptin in aP212. Insulin (1 unit/day/mice) or equal volumes of saline were given subcutaneously to controls and transgenic aP212 mice for two weeks. Plasma leptin was assayed by RIA. Average plasma leptin levels $\pm$ S.E. of transgenic and control mice. * vs. control mice, $P<0.05$.

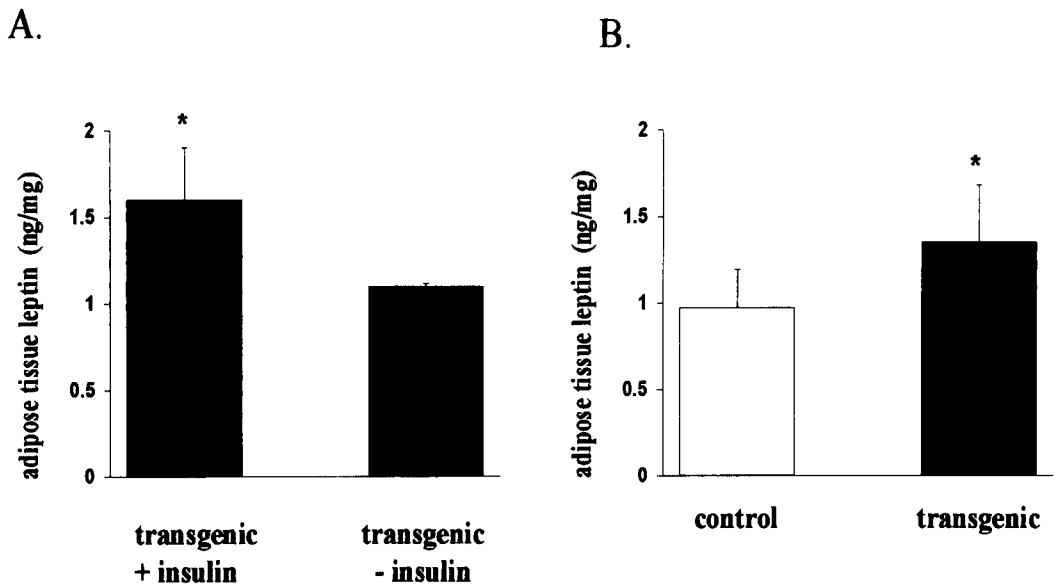

Figure 3. Effects of insulin administration on adipose leptin content in mice bearing adipocyte-specific agouti expression. Animals were treated as described in the legend for figure 2. A). Effect of agouti expression in adipose tissue on tissue leptin content. Adipose tissues leptin was assayed by RIA. * Insulin treated transgenic vs. saline treated transgenic mice; $P<0.05$. (B). Average adipose tissue leptin levels $\pm$ S.E. of transgenic and control mice (conbimation of insulin and saline treatments). * vs. control mice; $P<0.05$.

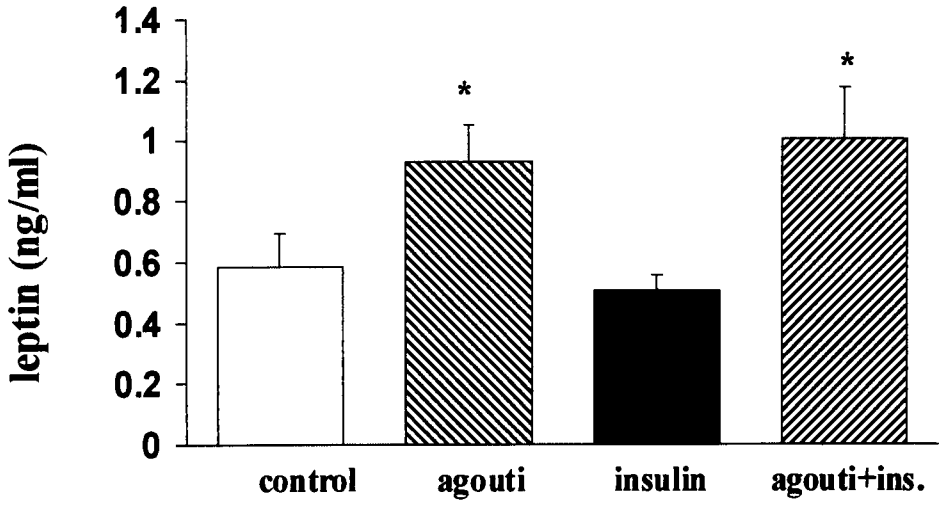

Figure 4. Effects of insulin and agouti on secreted leptin levels in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were incubated overnight in serum free medium followed by treatment with agouti protein (100 nM) and/or insulin (100 nM) for 24 hours. Leptin concentration in the culture media was measured by RIA. * vs. control; $P<0.05$.

$p<0.05$) while insulin showed no effect (0.51 ± 0.05 ng/ml). Addition of agouti and insulin caused similar increase (1.0 ± 0.17 ng/ml) to that caused by agouti alone (Fig. 4).

E. Discussion

Several recent studies have investigated insulin regulation of leptin, but inconsistent results have been reported. Some investigators have suggested that insulin treatment significantly increased leptin secretion (20-22) while others showed no significant effect of elevated insulin levels on plasma leptin content (23-25). Pharmacological doses (700 nM) of insulin were used to induce increased leptin mRNA and plasma levels, in vitro (20), however, the effects of this high insulin concentration might be mediated through insulin-like growth factor-1 receptors. Inability of insulin to increase plasma leptin levels (Fig. 2), may have resulted from increased clearance of circulating leptin or its binding to other proteins. The possibility that insulin may have an effect on packaging and secretory processes, which are not the main focus of this work but clearly need 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, no significant relationship between body fat and plasma leptin levels were observed (25).

Previous studies demonstrated that yellow obese mice exhibit elevated levels of plasma leptin levels (9). Similarly, BAP20 transgenic mice, who recapitulate the yellow mouse obesity syndrome (17), also express very high levels of plasma leptin (Ref. 15 and

Fig. 1). This increase in plasma leptin is paralleled by a significant increase in adiposity in these models (9, 10, 15). Although, aP212 transgenic mice exhibit approximately two-fold increase in plasma leptin levels compared to their control littermates; the body weight and fat pad weights 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 (26), showed that increased leptin levels are associated with subsequent weight gains and adiposity, and suggested that obesity in this population is associated with leptin resistance and is preceded by increased leptin levels. In agreement with these studies, recent reports indicated that hyperleptinemia may be an early marker of juvenile obesity (27). Since aP2 mice do not express agouti in the brain and agouti does not circulate, this indicates that the melanocortin and leptin signaling are intact. However, 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 agouti-induced obesity.

In summary, our current findings demonstrate that agouti directly upregulates adipose tissue leptin as well as plasma leptin levels. Since agouti is normally expressed in human adipose tissue (28), 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.

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II. INSULIN INCREASES FATTY ACID SYNTHASE GENE TRANSCRIPTION IN HUMAN ADIPOCYTES[1].

A. Abstract

The purpose of this study was to investigate the molecular mechanism whereby insulin increases expression of a key de novo lipogenic gene, fatty acid synthase (FAS) in cultured human adipocytes and hepatoma cells. RNA isolated from cultured adipocytes or from HepG2 cells treated with or without insulin (20 nM) was analyzed. In addition, run on transcription assays and measurement of RNA half-life were performed to determine the controlled step in FAS gene regulation by insulin. We demonstrated that FAS mRNA was expressed in both HepG2 cells and human adipocytes. Insulin induced an approximate 5- and 3- fold increase in FAS mRNA content in adipocytes and hepatoma cells, respectively. Similar regulation of FAS was observed in adipocytes from lean and obese human subjects. Furthermore, we demonstrated that the induction of human FAS expression by insulin was due to increased transcription rate of the FAS gene in human adipocytes while mRNA stabilization accounted for increased FAS mRNA content in hepatoma cells. In conclusion, we report here for the first time expression of human FAS mRNA and its specific transcriptional induction by insulin in cultured human adipocytes.

[1] This manuscript has been published in similar form with authors Claycombe, K.J., Jones B.H., Standridge, M.K., Guo, Y., Chun, J.T., Taylor, J.W., and Moustaid-Moussa, N. in: Am. J. Physiol. 1998 May; 274(5 Pt 2):R1253-9.

B. Introduction

Adipose tissue is the major site for energy storage and plays an important role in maintaining glucose homeostasis (12). Abnormalities in hormonal and nutritional regulation of this tissue have been implicated in the pathophysiology of obesity, diabetes and atherosclerosis. It is therefore crucial to investigate regulation of adipose tissue metabolism, in particular that of human adipose tissue.

While the causes of human obesity are not yet elucidated, this disease is commonly associated with excessive fat storage leading to adipocyte hypertrophy. In addition, obesity is highly prevalent in type II diabetic patients and diabetic patients on insulin therapy tend to gain weight. Therefore, the objective of this study was to investigate the role of insulin in regulating fatty acid synthesis in human adipose tissue.

Several studies have demonstrated the presence of the key enzymes for fatty acid synthesis (4, 23) and importance of human adipose tissue in de novo fatty acid synthesis (2, 5). In addition, recent studies demonstrated that liver plays a minor role in de novo human lipogenesis, and suggested that adipose tissue may be the principal lipogenic tissue in humans (1). As an approach towards understanding the role that nutrients and hormones play in regulation of human adipose tissue metabolism, we recently developed a cell culture system in which human adipocytes can be maintained viable and metabolically active for several days (21). We have demonstrated that glucose utilization as well as activities of lipogenic enzymes including fatty acid synthase (FAS) were increased by insulin in a dose-dependent manner in cultured human adipocytes (21).

FAS is a key lipogenic enzyme that catalyzes all of the reactions involved in the

synthesis of long chain saturated fatty acid (palmitate) from acetyl CoA, malonyl CoA and NADPH. In addition, this enzyme is highly regulated by nutrients and hormones in all species tested. In rodent and murine cell lines, FAS is suppressed by fasting (25), polyunsaturated fatty acids (8) and diabetes (25) while induced by feeding high carbohydrate diets (25), glucose (10), obesity (9) and insulin (25). We have recently identified an insulin response sequence in the proximal promoter of the rat FAS gene (20) that mediates regulation of FAS gene transcription by insulin. This element overlaps the CAAT box region and binds upstream stimulatory factor (29). A second insulin response element has been identified within a DNase hypersensitive region (30). Although regulation of human FAS gene serves as a better tool towards our understanding of lipid metabolism disorders in human obesity and diabetes, very limited information is available concerning its regulation. Human FAS has been shown to be expressed in several human tissues (14, 26). Semenkovich and his collaborators have reported that FAS expression was induced by glucose in HepG2 cells at the post-transcriptional level (26, 27). However, neither nutritional nor hormonal regulation of FAS gene expression in human adipocytes has been reported before.

Since our previous study showed induction of human lipogenic enzymes including FAS by insulin (21), the objective of the present work was to determine the mechanism(s) whereby insulin regulates expression of the FAS gene in cultured human adipocytes from lean and obese patients as well as in HepG2 human hepatoma cells. We report for the first time that FAS expression was induced by insulin at the transcriptional level in human adipocytes and at the posttranscriptional level in HepG2 cells.

C. Materials and methods

Human subjects: Non-obese, non-diabetic (BMI <27) as well as morbidly obese women (BMI>34) with an average age of 37 $\pm$ 15 years were used in this study. To our knowledge, these patients did not exhibit any other disorders or diseases. These patients required or elected abdominal surgery or liposuction. No information was available on the medications that the patients were taking or on their dietary habits or lipid profile prior to surgery. We maintained adipocytes isolated from these patients in culture for several days to eliminate any differences contributed by in vivo circulating factors. This proposal was approved by the institutional review board for human subjects and the Committee for Research Protocols of the University of Tennessee in Knoxville.

Isolation of human adipocytes and culture conditions: Adipose tissue was obtained from subcutaneous abdominal fat of the above fasted patients and cultured as we recently described (21). Fat was removed at the time of the surgery in a sterile environment and immersed in Hanks' balanced salt solution supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and gentamicin (50 μg/ml). Adipose tissue was washed several times with Hanks' balanced salt solution (Gibco, MD) followed by removal of the majority of connective tissue and blood clots. The tissue was minced into small fragments which were digested with type I collagenase (1 mg/ml, Gibco) in a shaking water bath at 37°C for 30 to 60 min. in a polypropylene flask. Cells were then filtered through a sterile nylon filter (350 μm mesh). The suspension was centrifuged at 500Xg for 1 min. to separate the pelleted stromal vascular fraction from the floating adipocyte fraction and washed three times with Hanks' balanced salt solution. Adipocytes were then resuspended in Dulbecco's

Modified Eagle's Medium (DMEM), supplemented with Hepes (15 mM), glucose (25 mM), bovine serum albumin (1%), 50 nM adenosine, and antibiotics; fetal bovine serum was added at a concentration of 1% (standard medium). Cells were subsequently cultured in suspension in sterile polypropylene tubes in a humidified incubator at 37°C under 5% CO_2 and 95% air. The culture medium containing the adipocytes (still in suspension) was removed 24 hrs later and the adipocytes were cultured in fresh standard medium. The cells were maintained for additional 3 to 6 days in this medium then incubated overnight in serum-free medium prior to insulin treatment as indicated in figure legends. Media were changed every day during the culture. Trypan blue exclusion test was conducted in all cultures to confirm cell viability.

Culture of HepG2 cells: HepG2 cells were purchased from ATCC (American Type Culture Collection, Bethesda, MD). Cells were grown and maintained in DMEM supplemented with 10% fetal bovine serum. Subconfluent cells were incubated overnight in serum-free medium prior to insulin treatment as indicated in figure legends.

FAS enzyme activity: Adipocytes were washed with phosphate-buffered saline (PBS) and homogenized in sucrose buffer then FAS activity was assayed spectrophotometrically as we previously described by measuring the rate of oxidation of NADPH (15). 1 unit of enzyme activity equals 1 nmol of NADPH oxidized/min/μg DNA that was assayed fluorometrically (3).

RNA isolation and hybridizations: RNA was isolated by the cesium chloride density gradient method and analyzed by Northern and/or dot-blotting (15, 22). A 2 kb human FAS cDNA probe, HFAS (27) was kindly provided by Dr. C.F. Semenkovich (St.

Louis, Mo). 18S rRNA probe was obtained from Clonetech laboratories (Palo Alto, CA). Sequential hybridizations with FAS, actin and 18S probes were conducted as we previously described (15, 22). Changes in FAS mRNA were normalized to 18S ribosomal RNA or to β-actin.

Nuclei preparation and nuclear transcription run-on assay: Nuclei preparation from human adipocytes has not been previously reported. We performed this preparation in various buffer conditions and have found that addition of very low concentrations of Nonidet P-40 (0.005%) to the cell lysis buffer containing 5 mM $MgCl_2$, 10 mM Tris, pH 7.5, 25 mM KCl, 0.1 mM EDTA and 1 mM DTT yielded satisfactory nuclei recovery. Adipocytes were first rinsed in PBS and resuspended in the above NP-40-supplemented lysis buffer. The homogenate was then centrifuged at 500Xg at 4°C for 5 min. and the nuclei pellet was recovered by pipetting through the solid fat layer and transferred to a fresh tube. The suspension was rinsed with the same buffer without NP-40 then centrifuged as above and resuspended in nuclei storage buffer containing 50 mM Tris, pH 7.8, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT and 40% glycerol. Nuclei were prepared form HepG2 cells as we previously described (22). Nuclear run on assay and hybridizations were conducted on both human adipocytes and hepG2 cells as we previously described for 3T3-L1 adipocytes (22). The following cDNA plasmids were used: pHFAS, human fatty acid synthase plasmid, kindly provided by Dr. C.F semenkovich, St. Louis, Mo (27), pFos plasmid, kindly provided by Dr. M.S. Miller, University of Tennessee, Knoxville (18) and pAR, angiotensin II type 2 receptor cDNA, kindly provided by Dr. T.S. Elton, University of Alabama at Birmingham (18).

mRNA stability: HepG2 cells were cultured as described above. Subconfluent cells were maintained overnight in serum-free medium then treated with insulin for 24 hours. Actinomycin D (5 :g/ml) was then added to cells as we previously described (22). Cells were harvested at different time points following actinomycin D treatment. RNA was then isolated and analyzed by Northern blot. Sequential hybridizations with FAS and 18S probes were conducted as we previously described (15, 22). The relative abundance of FAS mRNA as a function of time was used to determine FAS mRNA half-life.

Data analysis: Autoradiograms from Northern and dot blot analyses and run on assays were quantified by densitometric scanning. Alternatively, membranes were counted using Ambis 4000 direct β-imaging system (Billerica, MA). Post-hoc comparisons between groups were made using student's t-test. Data are expressed $\pm$ SEM.

D. Results

We have recently described a cell culture system of human adipocytes in which glucose consumption and activities of lipogenic enzymes were increased by insulin (21). In these studies, we have also shown that insulin increases FAS activity in a dose-dependent manner with a maximal induction at less than 10 nM. Furthermore, FAS activity was increased by insulin within 3 days of treatment (21). In the present work, we investigated molecular mechanisms whereby this key lipogenic gene was regulated by insulin in cultured adipocytes from lean and obese patients.

In preliminary studies, we first measured FAS mRNA levels in adipose tissue from different patients; these studies indicated expression of a single mRNA species of

approximately 9.3 kb in human adipose tissue (data not shown). This size is about 1kb larger than that previously reported for other species but comparable to that reported in HepG2 cells (26, 27). Since adipocytes have been removed from their in vivo environment where patients may exhibit differences in circulating factors, any differences obtained in culture would represent intrinsic properties of these cells, independently of the in vivo conditions. Expression of human FAS mRNA exhibited large variations at basal levels and this variation may have resulted from differences in dietary intake and other factors such as medications. To alleviate these variations, we cultured human adipocytes for several days to allow for their desensitization to in vivo circulating factors. Previous studies in Zucker rats have shown that the differences observed in adipocytes, freshly isolated from lean and obese animals, which were fed high fat diets, disappeared when adipocytes were maintained a few days in culture (6).

To investigate effects of insulin on FAS expression, we cultured cells for 4 to 7 days in standard medium, followed by an overnight incubation in serum-free medium prior to insulin treatment. Adipocytes were subsequently maintained with or without 20 nM insulin for an additional 2 to 4 days. We analyzed RNA from adipocytes of 11 non-obese and 6 morbidly obese patients. No statistical difference was observed between data form lean and obese patients. Therefore, we combined data from lean and obese patients and reported for each patient differences in insulin stimulation compared to control (untreated) cells. Preliminary experiments indicated that optimal responsiveness of FAS mRNA to insulin was reached after 48hrs. of treatment. No further stimulation was obtained at 60 hrs. or 72 hrs. Therefore, we treated cells for RNA analysis for 48hrs.

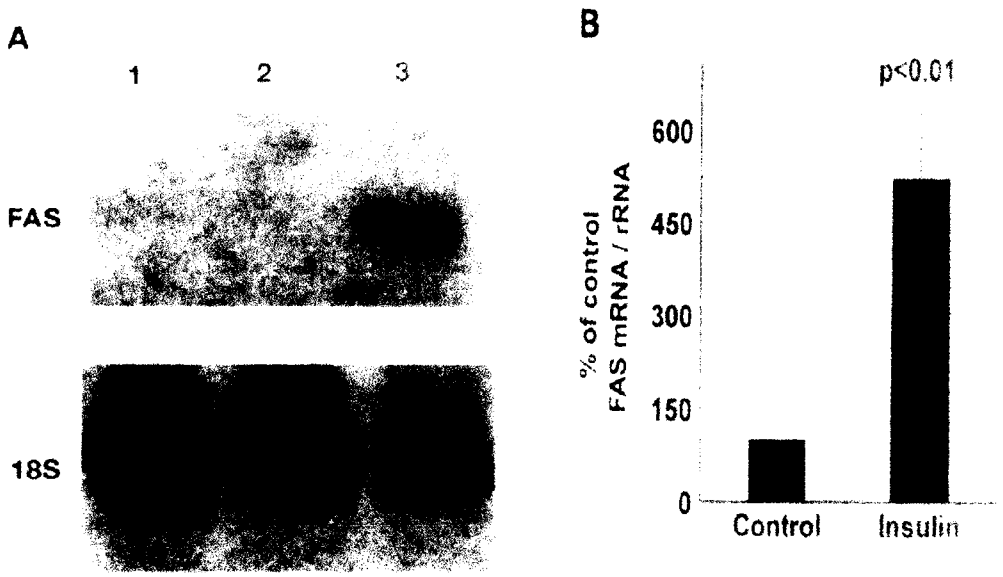

Figure 1. Insulin increases FAS expression in human adipocytes. Total RNA was isolated from human adipocytes treated with or without insulin (20 nM) for 3 days. 1A. 20μg RNA was analyzed by Northern blot; Hybridization with human FAS (FAS) and ribosomal (18S) probes are shown in this representative northern blot. 1. freshly isolated adipocytes; 2. adipocytes cultured without insulin; 3. Adipocytes cultured with insulin. 1B. Autoradiography obtained form Northern and/or dot blot analysis of RNA isolated from adipocytes of lean and obese patients (n=17) were analyzed by densitometric scanning. Data are expressed ± SEM, after normalization to 18S ribosomal RNA.

Fig. 1A is a representative autoradiogram of Northern blot analysis of human adipocyte RNA hybridized with human FAS and 18S RNA probes. Results from this Northern blot analysis indicated low basal level expression of FAS mRNA in human adipocytes and increased mRNA content upon insulin treatment. Densitometric scanning of data obtained from 17 subjects are shown in fig. 1B and indicate that insulin increased FAS mRNA levels by approximately 5-fold in adipocytes from lean and obese patients ($p<0.01$). This increase was paralleled with similar increase in FAS activity (0.073+0.011 units, control, vs 0.32+0.07, insulin, $n=10$, $p<0.01$). We further investigated whether continuous presence of insulin was required to sustain levels of FAS expression and whether the effect of insulin was reversible. Adipocytes were cultured for three days (day 0-3) in insulin-free and serum-free medium then either maintained in the same medium or treated with insulin for three additional days (day 3-6). As shown in fig. 2A, insulin-treated cells (C) exhibited higher FAS activity compared to control cells (A). Deprivation of insulin for three days of insulin was reversible and that continuous presence of insulin was required for induction of FAS activity. We also measured total DNA content in insulin-deprived (B) and insulin-treated cells (C) and found no significant difference between the two groups, suggesting that insulin at 20 nM induces cell hypertrophy rather than hyperplasia in human adipocytes. In agreement with the enzyme activity data in fig. 2B, FAS mRNA levels were lower in insulin deprived cells (-) compared insulin-treated cells (+). Addition of insulin to previously deprived cells (-/+) increases FAS mRNA content, while withdrawal of insulin from insulin-treated cells (+/-) decreases FAS mRNA content to levels observed in cells that were never exposed to insulin (-). Insulin is a

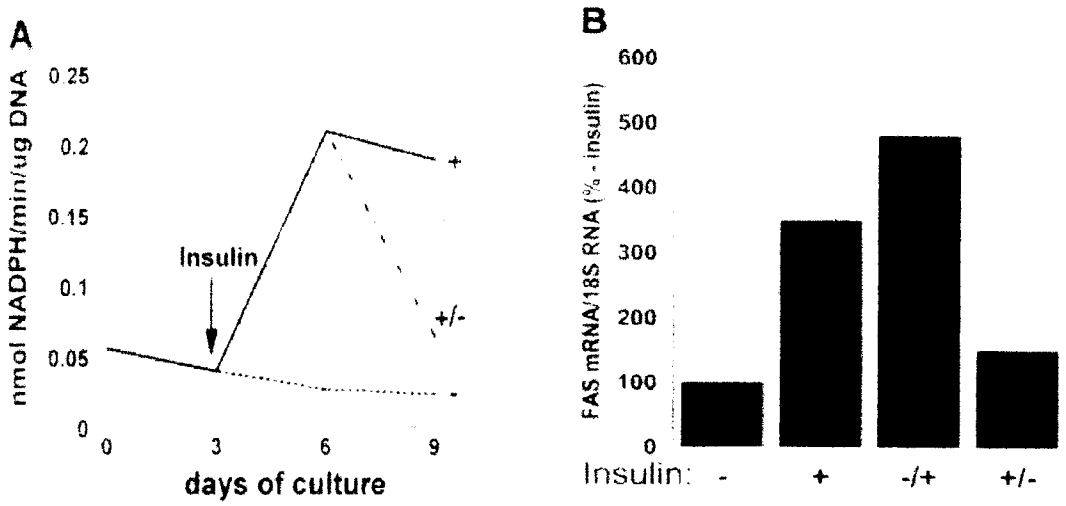

Figure 2. Continuous presence of insulin is required to maintain high levels of FAS. 2A. Human adipocytes were cultured without insulin for three days (day 0-3) then maintained without or with 20 nM insulin for additional three days (day 3-6). At day 6, cells were either deprived or maintained in the presence of 20 nM insulin. Cells were harvested and FAS activity was measured at the indicated times. This experiment was repeated twice. 2B. Human adipocytes were cultured as described above then maintained without (-) or with 20 nM insulin (+) for two days. Cells deprived from insulin (-) were then treated with the hormone (-/+) for two additional days while cells previously treated with insulin (+) were deprived from the hormone for two additional days (+/-). (day 6-9).

lipogenic hormone known to increase triglyceride stores; consistent with these effects, we have also recently reported that insulin slightly increases cell size in human adipocytes (21).

Our results thus demonstrate that adipocytes from both lean and obese patients are responsive to insulin, which induces FAS gene expression in these cells when continuously exposed to the hormone. Similar induction of the lipoprotein lipase mRNA by insulin has been recently reported in cultured human adipose tissue fragments (11). In addition, similar upregulation of the FAS mRNA by insulin has been previously reported in 3T3-L1 adipocytes (24).

In order to determine whether regulation of expression of FAS by insulin was tissue specific, we compared regulation of this gene in adipocytes and HepG2 cells. Recent studies have reported that FAS gene was post-transcriptionally regulated by glucose in HepG2 cells (26, 27). However, regulation of FAS gene in these cells by insulin has not been reported. Accordingly, we analyzed RNA isolated from HepG2 cells treated for 48 hrs with or without 20 nM insulin. A representative Northern blot is shown in fig. 3A. Results shown in this figure indicate that human FAS is expressed in HepG2 cells and that expression of this message is induced upon insulin treatment. Data from densitometric scanning of three independent measurements are shown in fig. 3B. These results indicate that insulin increases FAS mRNA content by an approximate 3- fold in human hepatoma cells. Taken together, the above data indicate that both hepatic and adipocyte FAS mRNA are upregulated by insulin. It is worthnoting that angiotensinogen mRNA was not significantly changed by insulin in HepG2 cells (Fig. 3A). Our findings in

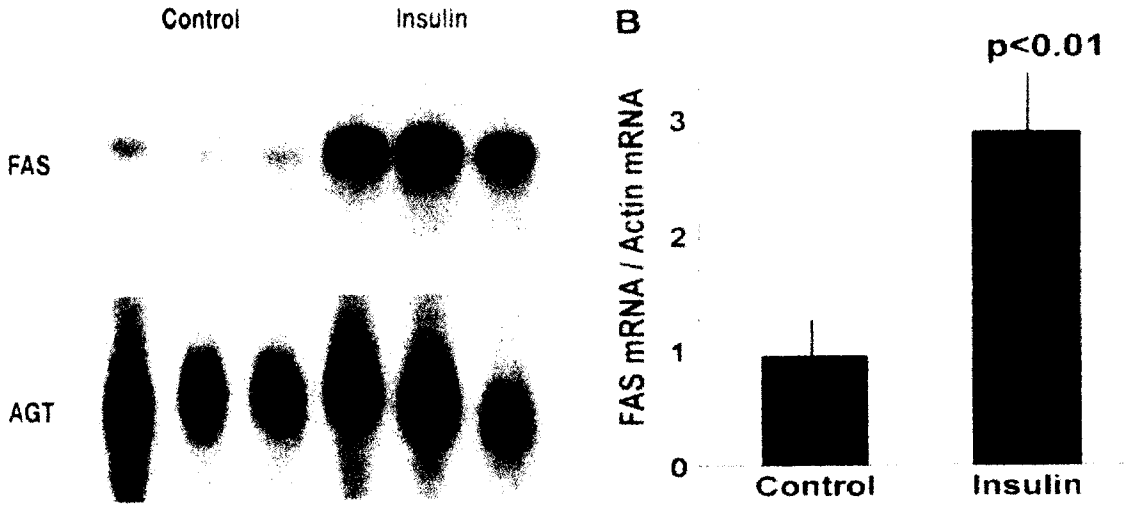

Figure 3. Insulin increases FAS mRNA content in HepG2 cells. Total RNA was isolated from hepatoma cells treated with or without insulin (20 nM) for 48 hrs. 3A: RNA were analyzed by Northern blotting after hybridization with HFAS and angiotensinogen (AGT) cDNA's as shown in this representative Northern blot. This experiment was repeated four times. 3B: Scanning of autoradiograms obtained from four independent experiments is shown after normalization to β-actin mRNA.

HepG2 cells are supported by other studies in H4 hepatoma cells, which demonstrated that glucocorticoid was the only hormone that regulates angiotensinogen expression in hepatoma cells. This is in contrast to previous reports suggesting that insulin is a key regulator of angiotensinogen expression in rodent adipose tissue (7). We have recently confirmed these finding by demonstrating that insulin increases angiotensinogen gene expression in cultured adipocytes (17). Taken together, these data suggest that insulin regulation of angiotensinogen may be tissue specific.regulator of angiotensinogen expression in rodent adipose tissue (7). We have recently confirmed these finding by demonstrating that insulin increases angiotensinogen gene expression in cultured adipocytes (17). Taken together, these data suggest that insulin regulation of angiotensinogen may be tissue specific.

To gain insight into mechanisms involved in regulation of FAS by insulin, we next investigated whether human FAS gene was regulated at the transcriptional level. Accordingly, we performed run on transcription assay in nuclei isolated from control and insulin-treated human adipocytes. These assays are difficult to perform in human adipocytes and require large amounts of cells. To our knowledge, this is the first report on nuclei isolation and measurement of transcription rate in human adipocytes. A representative assay is illustrated in Fig. 4A and scanning of autoradiograms from independent run on assays performed in 5 different patients are presented in Fig. 4B.

Data from Fig. 4B show that the transcription rate of the human FAS gene was increased by approximately 4-fold in insulin-treated compared to control untreated adipocytes. Similar results were obtained for lean and obese patients. Time course

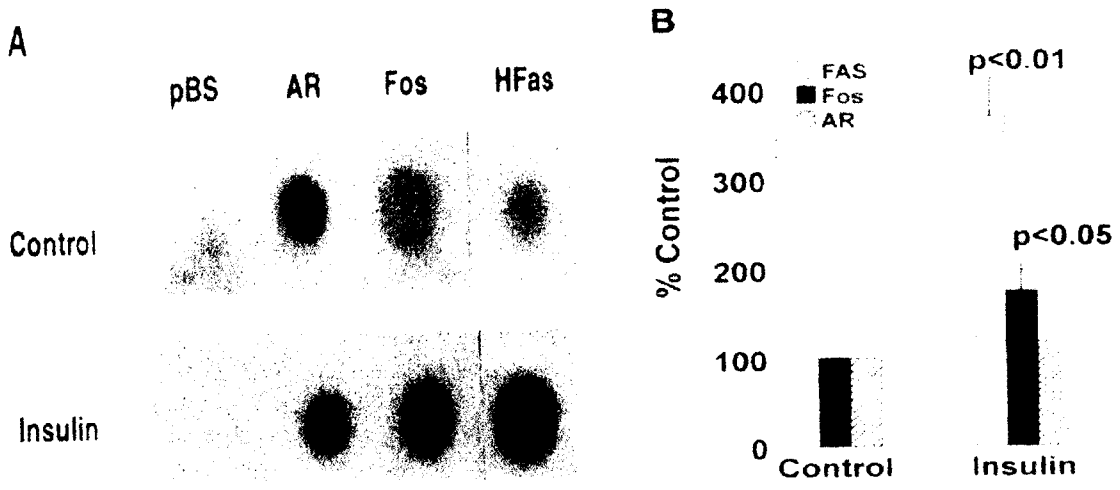

Figure 4. Insulin induces the transcription rate of the fatty acid synthase gene in human adipocytes. Nuclei were isolated from human adipocytes treated for 48 hours with or without 20 nM insulin. RNA were labeled with ^{32}P-[UTP], purified and hybridized with cDNA plasmids immobilized on "gene screen" nylon membranes. 4A: A representative autoradiogram is shown. 4B: Densitometric scanning of run on assays performed using hFAS (n=5), pAR (n=2) and pFos(n=3). The cDNA plasmids used were: pBS, BlueScript control plasmid; pHFAS, human fatty acid synthase plasmid; pFos, Fos oncogene plasmid and pAR, angiotensin II type 2-receptor plasmid.

experiments indicated that insulin had no effect on FAS gene transcription in human adipocytes treated for 6 hrs. while insulin effect at 72 hrs was lower but not significantly different form 24 or 48 hrs. (Data not shown). To demonstrate specificity of the insulin effect, Angiotensin II type 2 receptor (AT2) and fos oncogene cDNA's were used as controls. Our results (Fig. 4) demonstrate that AT2 gene transcription was not significantly changed by insulin [range of insulin effect was –23% to +18%]. We have recently reported that angiotensin II type receptor (AT2) antagonist, PD123319 antagonized lipogenic effect of angiotensin II in murine 3T3-L1 adipocytes (16). However, this is the first report of expression of this receptor in human adipocytes. We also report that fos oncogene transcription (used as a positive control) was also induced by insulin in human adipocytes; this finding is in agreement with previously reported induction of the fos gene transcription by insulin in 3T3-L1 adipocytes (28). Interestingly, when nuclear run on assays were performed in HepG2 cells, no significant difference was observed on FAS gene transcription in control compared insulin-treated cells (data not shown).

Previous reports in rodents have demonstrated that the rodent FAS gene was primarily regulated at the transcriptional level in liver and adipose tissue as well as in hepatoma cells and preadipocyte cell lines (9, 20, 25). Interestingly, our study demonstrated that transcriptional regulation of the human FAS gene by insulin is tissue specific since insulin did not affect FAS gene transcription in HepG2 cells. However, FAS gene transcription was increased by insulin in rat hepatoma cells H4IIE cells (20) suggesting specie specific differential regulation of the FAS gene by insulin.

To further support our findings, we used actinomycin D to determine whether FAS mRNA stability was modified by insulin in HepG2 cells. Since FAS gene transcription accounted for changes in FAS mRNA levels in adipocytes, the message stability was only investigated in HepG2 cells. The relative abundance of FAS mRNA levels were measured in HepG2 cells, which were treated with insulin for 24 hrs. prior to addition of actinomycin D for up to 24 hrs (n=3). Results from densitometric scanning analysis of the autoradiograms (Fig. 5) indicated that FAS mRNA content declined more rapidly in control compared to insulin-treated cells. The half-life of the FAS mRNA was estimated to be about 3 hrs in controls versus 15 hrs in insulin-treated cells. This FAS mRNA half-life is lower but comparable to that previously reported in HepG2 cells (27), which estimated FAS mRNA half-life to 4.4 hrs. in the absence of glucose and 30 hrs. in the presence of glucose. The shorter half-life in our studies may be due to long term incubation of cells in serum-free medium. Nevertheless, both our findings and the above report (27) demonstrate that changes in FAS expression in HepG2 cells are not due to changes in the transcription rate of this gene but rather due to the stabilization of the message.

E. Discussion

Taken together these finding suggest that regulation of human FAS may be different in liver compared to adipose tissue, however, this does not overrule the possibility that this difference may be due to the transformation and malignancy of HepG2 cells.

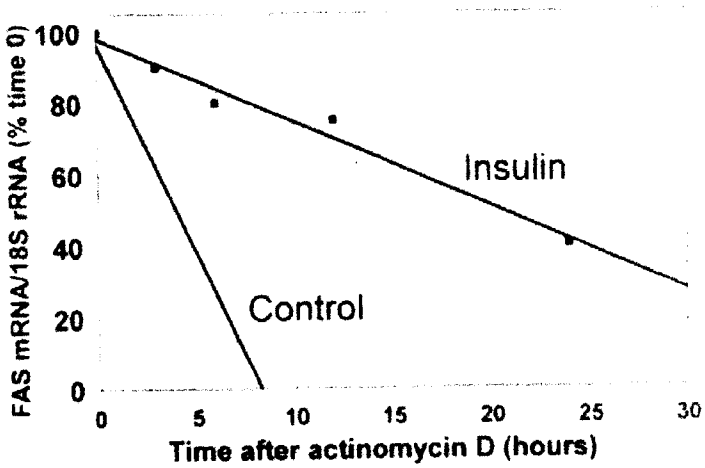

Figure 5. Insulin stabilizes FAS mRNA in HepG2 cells. Subconfluent HepG2 cells were maintained overnight in serum-free medium then treated with or without insulin (20 nM) for 24 hours prior to addition of actinomycin D (5 g/ml) for up to 24 hrs. Cells were harvested at the indicated times and RNA was isolated and analyzed by Northern blot using FAS and 18S probes as indicated in the methods section. This figure is representative of three separate experiments.

Mechanisms of post-transcriptional regulation of FAS have been recently demonstrated in HepG2 cells (26, 27). These studies showed that glucose regulates cytoplasmic FAS mRNA by partitioning the message between a translated pool not subject to degradation and a decay compartment. However, mechanisms involved in transcriptional regulation of human FAS gene remains to be investigated. Identification of cis-acting elements in the 5' flanking region of the human FAS gene will allow us to determine whether an insulin response element(s) similar to that we previously identified in murine 3T3-L1 adipocytes (20) mediates insulin responsiveness of FAS in human adipocytes. HSu et al. recently reported the partial sequence of the human FAS promoter (13). Unlike the FAS gene sequence reported in other species, two differentially regulated promoters have been identified in human FAS gene. These studies have shown that transcription from human FAS upstream promoter is blocked by the intron promoter resulting in reduced overall expression. Consequently, it would of interest to investigate the functional characteristics of these promoters in human adipocytes and to determine whether they are involved in insulin regulation of the human FAS gene.

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III. TRANSCRIPTIONAL REGULATION OF THE ADIPOCYTE FATTY ACID SYNTHASE GENE BY THE AGOUTI GENE PRODUCTS: INTERACTION WITH INSULIN[1].

A. Abstract

Mice carrying dominant mutations at the agouti locus exhibit ectopic expression of agouti gene transcripts, obesity and type II diabetes through unknown mechanims. To gain insight into the role of agouti protein in modulating adiposity, we investigated regulation of a key lipogenic gene, fatty acid synthase (FAS) by agouti alone and in combination with insulin. We previously reported that both agouti and insulin increase FAS activity in 3T3-L1 adipocytes. Here we report that agouti and insulin independently and additively increase FAS activity in 3T3-L1 and human adipocytes. We further investigated the mechanism responsible for the agouti-induced FAS expression and demonstrated that both insulin (3-fold increase) and agouti (2-fold) increased FAS gene expression at the transcriptional level. Furthermore, insulin and agouti together exerted additive effects (5-fold increase) on FAS activity and gene transcription. Transfection assays of FAS promoter-luciferase fusion gene constructs into 3T3-L1 adipocytes indicated that agouti response element(s) is (are) located in the –450 to -395 region of the FAS promoter. Thus the agouti response sequences mapped to a region upstream of the insulin responsive element (which we previously reported to be located at -67-52), consistent with additive effects of these two factors on FAS gene transcription.

[1] This manuscript has been submitted for publication in similar form with authors Claycombe, K.J., Jones, B.J., Guo, Y.S., Wilkison, W.O., Zemel, M.B., Chun, J., and Moustaïd-Moussa, N. in: J.Clinc. Invest.

B. Introduction

Agouti, a paracrine factor composed of 131 amino acids, is normally secreted within hair follicles during the hair growth period (1). Secreted agouti protein regulates hair pigmentation by competitive antagonism of α-melanocyte stimulating hormone at its receptor (MC1-R), resulting in a switch from eumalanin to phaeomelanin production (2,3). Promoter mutations at the agouti locus resulting in ectopic expression of the agouti gene cause yellow coat color, marked obesity, hyperinsulinemia, type II diabetes (4) and development of cancer (5).

Although agouti was the first obesity gene to be cloned (6), the role of agouti in the development of obesity is not fully understood. Both peripheral and central effects of agouti have been implicated in yellow mouse obesity. Centrally, agouti has been shown to antagonize the hypothalamic melanocrtin receptor (MC4-R) resulting in inhibition of feeding behavior, possibly through neuropeptide Y (NPY) (7). In addition, we have obtained evidence supporting peripheral actions of agouti in the etiology of yellow mouse obesity. Fatty acid synthase (FAS) is expressed at significantly higher levels in the fat tissues of A^{vy}/a mice (viable yellow) compared to lean control (8) and in transgenic mice which ubiquitously overexpress the agouti gene (9). We also have shown that agouti increases FAS expression and lipogenesis in vitro via a $[Ca^{2+}]_i$-dependent mechanism (8,10). Further, insulin treatment of transgenic mice expressing the agouti gene specifically in adipocytes under the control of the aP2 promoter (11-13) causes a significant increase in body weight compared to untreated transgenic mice (14). Consequently, we

hypothesized that agouti interacts with insulin to induce obesity by upregulating lipogenesis in adipose tissue.

To gain insight into the specific role of agouti protein in adipocytes and its interaction with insulin, we investigated the effects of these two factors on FAS expression in adipocytes. FAS plays a key role in long term regulation of lipogenesis and catalyzes de novo synthesis of palmitate from acetyl-CoA, malonyl CoA(15). This pathway is highly regulated by nutritional factors as well as hormonal signals (16,17). Among hormonal inducers, insulin has been shown to induce FAS in several species (9, 17-21). We have previously mapped the insulin response element within the proximal rat FAS promoter (22).

In our present study, we report an additive effect of agouti and insulin in inducing FAS activity in 3T3-L1 and primary human adipocytes. Furthermore, this induction of FAS activity is due to increased FAS gene transcription in 3T3-L1 adipocytes. Finally, we map the agouti response element to a region distinct from the insulin response element.

C. Materials and methods

Expression of agouti cDNA: Full-length agouti cDNA was subcloned into a baculovirus expression vector and expressed in Trichiplusia ni cells. Medium was collected 48 hrs after infection and partially purified as previously described(2).

3T3-L1 cell culture: 3T3-L1 cells were grown and differentiated as previously described (23,24). Briefly, cells were grown to confluence in standard medium (Dulbecco's modified Eagle's medium, DMEM supplemented with 10% fetal bovine

serum, FBS). At confluency, cells were induced to differentiate by addition of dexamethasone (250 nM) and iso-butylmethylxanthine (0.5 mM) to standard medium for two days. Cells were maintained for 3 additional days in standard medium, then changed to serum free medium (containing 1% BSA) followed by treatment with agouti protein and/or insulin as indicated in the figure legends.

Human adipocyte cell culture: Human adipose tissue was obtained from abdominal fat of patients undergoing elective liposuction or abdominoplasty. These tissues were processed as previously described (20, 21). Briefly, adipose tissue was minced into small fragments, digested with collagenase (Gibco/BRL, Bethesda, MD), filtered (250-300 μm mesh), and centrifuged to separate the adipocyte fraction. Adipocytes were maintained in DMEM medium supplemented with 1 % FBS and cells were treated as described in figure legends.

FAS activity: FAS activity was measured spectrophoto-metrically in crude cytosolic extracts of 3T3-L1 or human adipocytes by measuring the oxidation rate of NADPH at 340 nm (25) per minute. FAS activity was normalized per mg protein, assayed by Bradford method or per ug DNA, assayed fluoremitrically as we previously described (21).

Nuclear run-off assay: Nuclei were isolated from control, agouti (50 nM)and/or insulin (20 nM) treated 3T3-L1 adipocytes as previously described (24). Nuclei were then labeled with ^{32}P-UTP and labeled RNA were purified and hybridized to rat FAS cDNA (kindly provided by Dr. A.G. Goodridge, Iowa) and LPL cDNA (kindly provided by Dr. S. Fried, Rutgers University, NJ) which were immobilized on nylon membrane. LPL

cDNA was used as a control in these studies.

FAS-Luciferase fusion gene construct: A fragment of the FAS 5'- flanking region spanning -2100 to +67 was generated by PCR using rat genomic DNA and subcloned into the pGL-basic vector (Promega). Various deletions of this fragment were subsequently generated using ExoIII/mung bean nuclease deletion (Stratagene, La Jolla, CA) or by PCR using previously published FAS promoter sequences (26). These FAS promoter-luciferase fusion constructs were used to transfect 3T3-L1 cells.

Transient transfections: 3T3-L1 cells were transfected with FAS-luciferase fusion gene construct (22) using the calcium phosphate-DNA coprecipitation method (kit purshased from Gibco/BRL, Bethesda, MD). Transfected cells were maintained overnight in serum-free medium before treating with agouti and/or insulin as indicated in the figure legends. SV-β-galactosidase fusion construct (Promega, Madison, WI) was co-transfected with the FAS constructs to normalize for the transfection efficiency. pGL2-control, which contains the SV40 promoter linked to the luciferase gene was used as a control to determine specificty of agouti effect on FAS promoter.

Luciferase and β-galactosidase assays: The cells were lysed in 100 mM Potassium phosphate pH 7.8, 0.2% Triton X-100 and 1 mM dithiothrietol and cytosolic extracts were used for luciferase activity. Luciferase activity was measured utilizing a luminometer (Berthold, Nashua, NH) and luciferase Assay kit (Tropix, Bedford, MA).

Gel-mobility shift assays: Nuclei were isolated as described above and used to prepare nuclear extracts as previously described (22). Two complementary single strands of 55 bp oligonucleotides containing FAS promoter sequences spanning –450 to –395

region were synthesized then annealed by incubating equal molar concentrations in 1 M Tris-HCl (pH 7.5), 5 M NaCl and 0.5 M EDTA at 65°C for 10 min followed by cooling to room temperature. The annealed oligonucleotides were 5'-end labeled with [(-^{32}P]ATP using T4 polynucleotide kinase (Promega). Gel shift binding assay were performed at room temperature in 2 :l of binding buffer (5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris-HCl (pH 7.5) and 0.25 :g/:l poly dIdC in 20% glycerol) for 30 min. Each reaction contained 50,000 cpm(1.75 pmol) of oligonucleotides and 2 :g of nuclear extracts. Unlabeled competitors were added to reaction mixtures containing nuclear extract and binding buffer for 20 min. at room temperature prior to addition of labeled oligonucleotides. Electrophoresis of DNA-protein complexes were conducted using a 3% nondenaturing polyacrylamide gel in 50 mM Tris-HCl, 45 mM boric acid and 0.5 mM EDTA. The gels were dried and exposed to x-ray film at –80°C.

Statistical analysis: One way analysis of variance (ANOVA) was used (SAS, Cary, N.C.) for statistical analysis. All data are expressed as mean +/- SEM.

D. Results

Effects of agouti and insulin on FAS activity: We have previously shown that agouti significanly increases FAS activity and triglyceride content in 3T3-L1 adipocytes (8). In addition, we and others have shown that insulin induces FAS expression (20, 27). However, mechanism(s) of agouti and insulin to directly regulate FAS is not known. For both 3T3-L1 (fig. 1) and primary human adipocytes (fig. 2), agouti and insulin each increased FAS enzyme activity by 2-3-fold compared to control cells. Addition of agouti

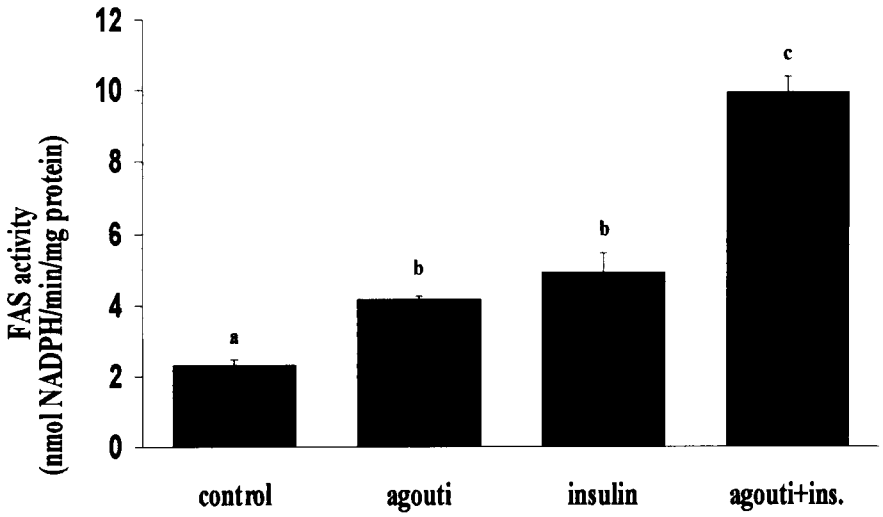

Figure 1. Effect of agouti and insulin on FAS activity in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were changed to serum free media and cultured overnight. Cells were maintained in serum free media during agouti (100 nM) and/or insulin (100 nM) treatment for 48 hrs and FAS enzyme activity was assayed as described in materials and methods. Data are presented as mean $\pm$ SEM. a, b, c: $p<0.01$; groups with different characters are statistically different from each other.

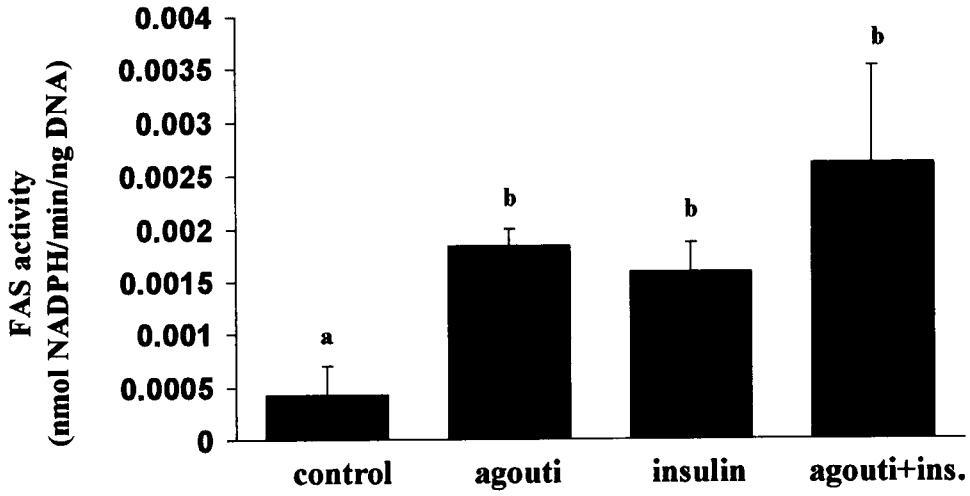

Figure 2. Effect of agouti and insulin on FAS activity in human adipocytes. Primary human adipocytes were cultured in 1% FBS supplemented media and treated with agouti (100 nM) and/or insulin (100 nM) for 48 hrs. Treated cells were harvested in 500 nM sucrose buffer and FAS enzyme activity was assayed as described in materials and methods. Data are presented as mean $\pm$ SEM. a, b $p<0.05$; groups with different characters are statistically different from each other.

with insulin caused approximately a 5-6-fold increase in FAS activity in 3T3-L1 adipocytes, demonstrating additive effects (fig 1). These data demonstrate that 1) consistent with our previous report, agouti, like insulin, directly regulates lipogenesis by inducing FAS activity, and 2) agouti and insulin independently and additively increase FAS activity.

Effects of agouti on FAS transcription levels: Increases in the levels of functional enzyme activity are normally correlated with increases in the amount of transcribed message and/or stability of message. We have recently shown that insulin regulates the adipocyte FAS gene primarily via transcriptional mechanisms (22). We therefore first tested whether increased FAS enzyme activity by agouti was due to increased FAS gene transcription. We performed nuclear run-off assays using nuclei isolated from agouti and/or insulin treated 3T3-L1 adipocytes and demonstrated that both insulin (3-fold increase) and agouti (2-fold) increased FAS gene expression at the transcriptional level. Agouti-induced FAS gene transcription was specific since transcription rate of lipoprotein lipase (LPL, a key gene in triglyceride accumulation in adipocytes) was not changed by agouti treatment (data not shown). Furthermore, a combination of these two hormones showed additive effects (5-fold increase) on FAS gene transcription rate (fig.3). These fold increases in the transcription rate (fig. 3) are consistent with those data observed in agouti induced FAS enzyme activity (figs. 1 and 2). These studies: 1. demonstarte that agouti, like insulin regulate FAS gene expression primarily at the transcriptional level and 2. suggest that insluin and agouti act via independent transcriptional elements/factors in the FAS gene .

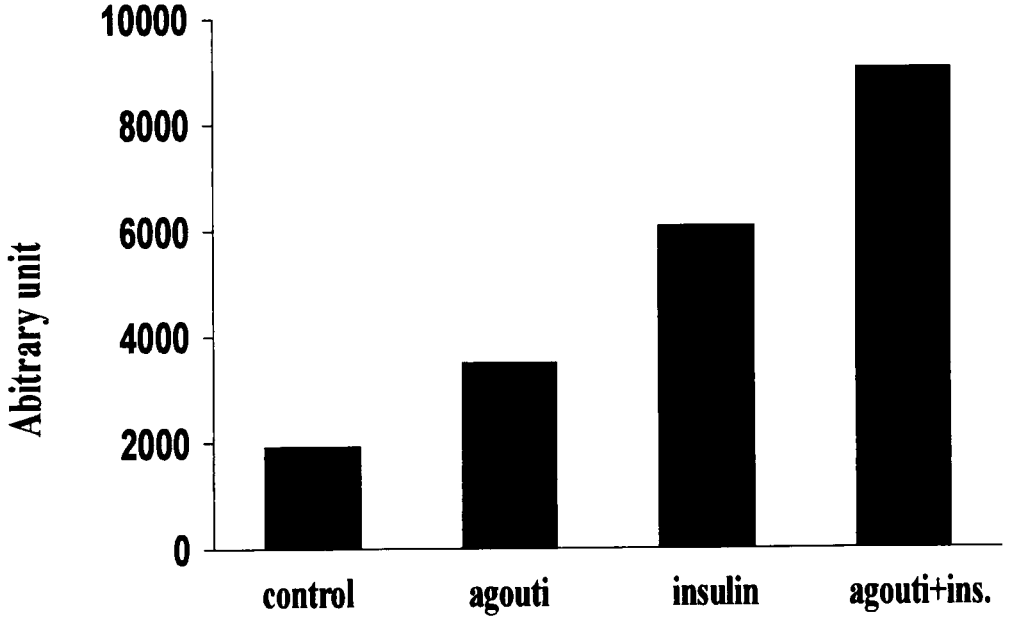

Figure 3. Effect of agouti and insulin on FAS gene transcriptional rate in 3T3-L1 adipocytes. Nuclei were isolated from 3T3-L1 adipocytes have been treated with agouti (50 nM) and/or insulin (20 nM) for 24 hrs. Nuclei were then labeled with ^{32}P UTP and hybridized to FAS cDNA, which was immobilized on nylon membrane, and radioactivity was counted as arbitrary unit. SV40 promoter sequence containing control DNA was used to normalize quantitative measurements in all nuclear run-off assays.

Agouti response element(s) in FAS promoter: To further investigate the mechanisms of transcriptional regulation of the FAS gene by agouti,we searched for agouti responsive region(s) within the FAS promoter. Using transfection assays, we initially tested a promoter region containing 500 bp of the FAS 5' flanking region. We have previously mapped the insulin response sequence within the FAS proximal promoter to -67 to -25 bp (22). We first transfected 3T3-L1 adipocytes with FAS-promoter luciferase fusion gene constructs (-500 to +67 and –300 to +67), both of which contain the insulin responsive region, and the effects of agouti on luciferase activity were measured. Agouti significantly increased luciferase activity (fig. 4A) in 3T3-L1 cells transfected with the –500 to +67 regions of the FAS promoter compared to cells transfected with –300 to +67 of the FAS promoter or pGL2-control, which were not responsive to agouti. To further narrow down the agouti responsive region, we generated deletion fragments within the fragment –500-300. In 3T3-L1 adipocytes transfected with a luciferase reporter construct linked to –450-395 region of the FAS promoter, agouti increased luciferase activity to levels comparable to those observed in cells transfected with –500+67 fragment(Fig. 4A). Other constructs spanning the –500-300 region were not responsive to agouti (not shown). These results indicate: 1. that agouti response sequences are located in the –450 to –395 region upstream of the FAS transcription start site, and 2. that the agouti responsive region is distinct from the insulin responsive region (22), indicating that these two factors regulate FAS gene transcription via different transcriptional mechanisms (figure 5).

Binding of nuclear factors to the putative FAS agouti response sequences: After

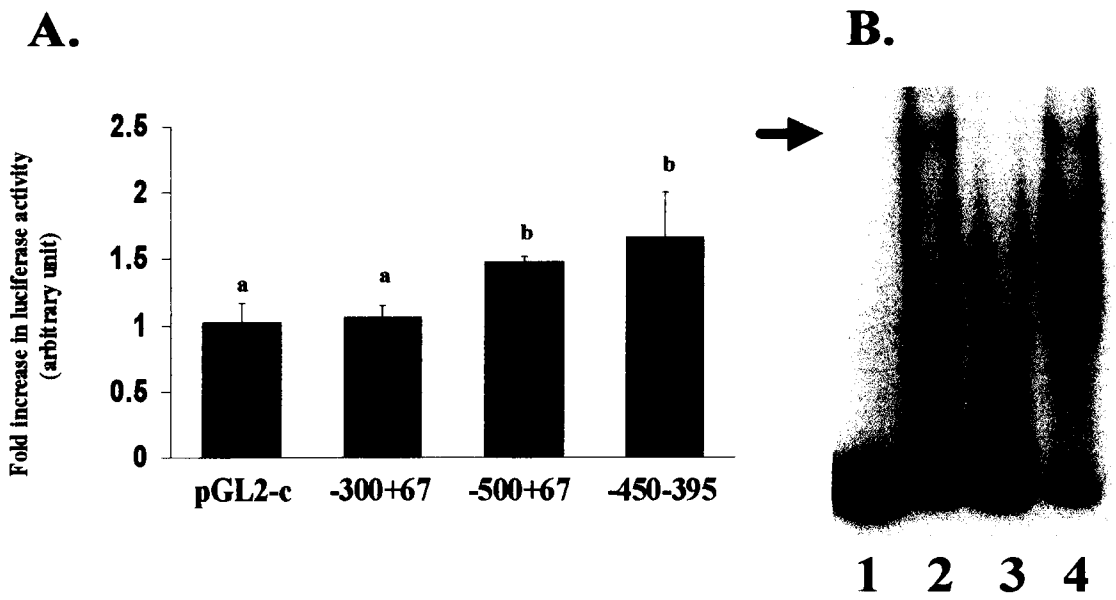

Figure 4. Agouti Response Element in FAS promoter. A. 3T3-L1 adipocytes were transfected with FAS-luciferase fusion gene constructs as described in methods. Transfected cells were maintained overnight in serum free media prior to treatment of agouti (100 nM) and/or insulin (100nM) for 48 hrs. Cells were then harvested and subjected to luciferase activity. Data are presented as mean ± SEM. a, b, $p<0.05$; groups with different characters are statistically different from each other. For the normalization of transfection efficiency, β-galactosidase gene (Promega, Madison, WI) containing control vectors were co-transfected. B. Gel mobility shift assay. Nuclear extracts of 3T3-L1 adipocytes were incubated with various unlabeled oligonucleotides in 5- fold excess followed by addition of ^{32}P labeled FAS promoter spanning region –450 and –395. ^{32}P labeled free oligo (-450 -395 FAS promoter); lane 1, ^{32}P labeled –450 -395 incubated with 3T3-L1 nuclear extract; lane 2, competition with unlabeled –450 -395 FAS promoter; lane 3 and competition with unlabeled SP1 oligo; lane 4. Arrow indicates the position of the specific gel mobility shifted complex.

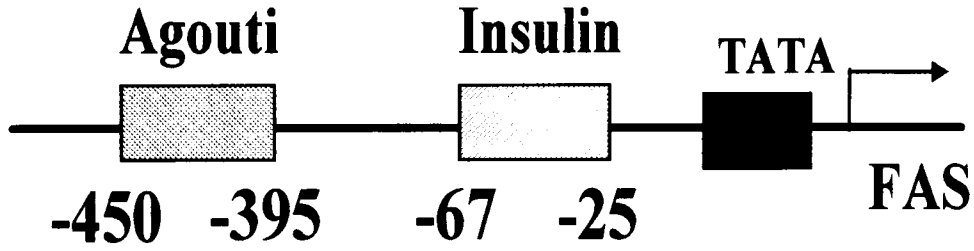

Figure 5. Insulin and Agouti Response Elements in FAS promoter.

we identified a 55 bp region of the FAS promoter contain the agouti response element(s), we used gel shift assays to demonstrate that nuclear factors present in agouti-treated 3T3-L1 adipocytes indeed bind to this region of the promoter. Nuclear extracts from 3T3-L1 adipocytes contained proteins that altered the electrophoretic mobility of oligonucleotides specific for the 55 bp region (-450 –395) {Fig. 4b, lane 2}. This major DNA-protein complex (indicated by the arrow) was competed away by unlabeled 55 bp oligonucleotides (-450 –395) {fig. 4b, lane 3}. In addition, unlabeled SP1 oligonucleotide showed no specific competion (fig 4b, lane 4), indicating specificity of this binding. These data indicate that this 55 bp region of the FAS promoter is the candidate site for agouti-mediated FAS transcription. It is worthnoting that, as described for other DNA-binding proteins, agouti treatment did not modify the binding capacity or mobility of this band. Importantly, nuclear proteins from both murine 3T3-L1 adipocytes as well as isolated human adipocytes were able to bind to this region (data not shown).

E. Discussion

We have mapped a novel agouti response region within the FAS promoter (-450 to -395) which is distinct from insulin responsive sequences (-67 to -52){fig. 5}. Insulin has been shown clearly to be a lipogenic inducer which acts directly on the FAS promoter. Several transcription factors that mediate insulin responsiveness have been identified; upstream stimulatory factor I (USF1) has been shown to bind to -71 to -50 position in the proximal promoter (28) and SP1 has been suggested to bind to -57 to -35 (29). However, transcription factor(s) induced by agouti protein are not known. To our knowledge, this is

the first report on regulation of gene transcription by agouti and of identification of agouti response sequences in that gene.

Agouti, like insulin, increases FAS activity in murine and human adipocytes. Using 3T3-L1 adipocytes, we demonstrated that this increase was mediated at the transcriptional level. Agouti and insulin together showed additive effects on FAS activity in both human and 3T3-L1 adipocytes, suggesting that these two factors act through independent transcriptin factors and DNA elements within the FAS promoter. This possibility is currently under investigation. The agouti response sequence reported here is novel sequence that binds more likely to a novel trans acting factor, since this sequence does not share homology with other published regulatory sequences. Future studies will use mutational analysis to fully characterise these regulatory elements. Interestingly, we have shown that yellow obese mice carrying agouti mutation exhibit increased $[Ca^{2+}]_i$ levels in several cell types, including adipocytes (9,32). Consistent with a Ca^{2+} -linked agouti transduction pathway, we have also shown that agouti induces FAS activity and cellular triglyceride content in 3T3-L1 adipocytes via a Ca^{2+} -dependent mechanism (8). Therefore, further studies will aim to determine whether this agouti response sequence serves also as a Ca^{2+} response element in the FAS regulatory region.

Agouti is normally expressed in human adipose tissue (30). Furthermore, we have recently shown that agouti is expressed in human pancreas where it serves as a potent insulin secretagogue (31). Thus these data indicate that the coexpression of agouti and hyperinsulinemia, often found in concert, may be attributable in part to combined actions

of agouti in adipose cells and in the pancreas.

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PART 4

CONCLUSIONS

SUMMARY AND CONCLUSIONS

In summary, the results from this study indicate that agouti effects on adipocytes possibly contribute to yellow mouse obesity. Agouti affects adipocyte metabolism by inducing synthesis and secretion of leptin, and by increasing FAS transcription rate via novel agouti responsive elements that are distinct from the previously mapped insulin response element. Preliminary data suggest that agouti response element is also the target of calcium.

Yellow obese mice express high levels of leptin and aP2 transgenic mice secrete significantly high levels of leptin compared to littermate control without developing obesity. We propose that Agouti-induced adipocyte leptin expression may limit the magnitude of agouti-induced obesity in obese yellow mice and may also be a predictor of obesity (in aP2 transgenic mice). Whether these effects are mediated via melanocortin receptor or by a yet unidentified agouti receptor remains to be investigated.

Additive effects of agouti and insulin on FAS transcription rate are important findings since humans express agouti in their adipose tissue and type II diabetic patients have elevated insulin levels. Physiological relevance of expression of agouti in human adipose tissue tested in "humanized" (express agouti in adipose tissue without mutation) transgenic mice supports the perspective that insulin and agouti interaction may a key factor in regulating adiposity. In addition to the lipogenenic effect of agouti and its potentiation of insulin effect, agouti also inhibits adipocyte lipolysis. Treatment of pancreatic β-cells with Agouti protein also increase insulin secretion. Therefore, it is plausible that a coordinate regulation of all of these factors modulate adipocyte

metabolism leading to increased adiposity (Fig. 1)

In conclusion, the present study defined molecular mechanisms whereby a single gene mutation modulate adiposity. However, this type of observation needs to be interpreted with caution since human obesity is a multifactorial disorder. Individual susceptibility to obesity is influenced by genetic inheritance, gene-gene as well as gene-environment interactions. All of which makes obesity research very challenging, and at the same time, very difficult (to determine precise causative mechanisms). The application of molecular and genetic techniques; however, provides exciting new insights into the physiological systems involved in metabolic regulation in obesity. As more information becomes available, potential new targets for therapeutic intervention will likely become evident.

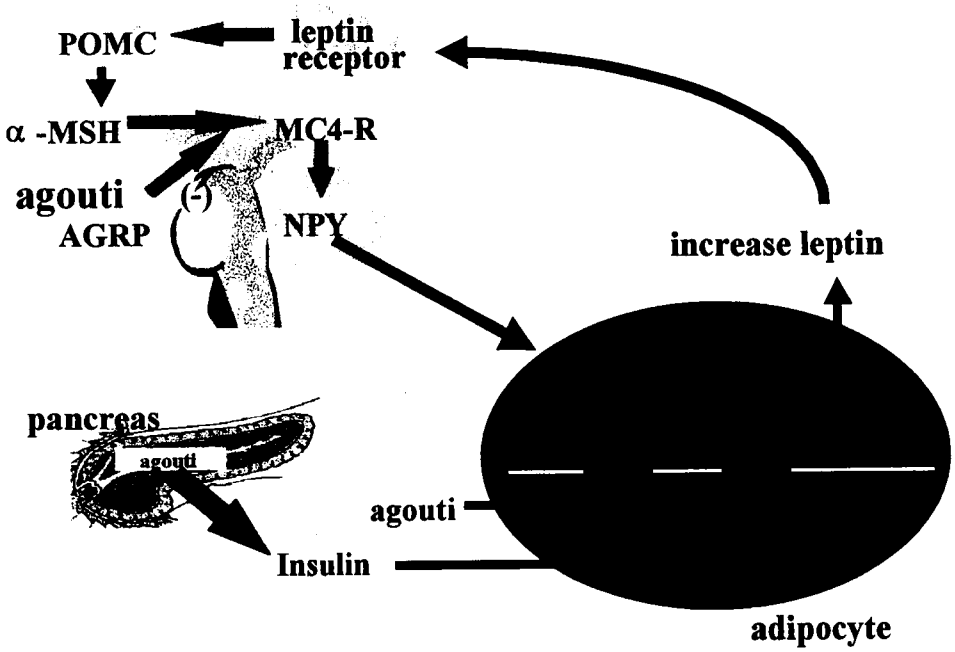

Figure 1. Mechanism of agouti-induced obesity: Effects on adipocyte metabolism and interaction with insulin. Agouti protein increases insulin secretion from pancreatic β-cells. Agouti (in combination with elevated levels of insulin) increases FAS gene transcription (through a novel agouti response element distinctly located from insulin response element), and inhibits lipolysis. These effects contribute to increased triacylglycerol levels, adipocyte hypertrophy and increaesd leptin secretion. Leptin binds to leptin receptor in the hypothalamus and inhibits food intake. Agouti and agouti related protein (AGRP) antagonize MC4-R and prevent leptin-mediated inhibition of food intake. Consequently, central and peripheral tissues coordinately control body fat stores.

APPENDIX

THE YELLOW MOUSE OBESITY SYNDROME AND MECHANISMS OF AGOUTI-INDUCED OBESITY[1].

A. Abstract

The yellow mouse obesity syndrome is due to dominant mutations at the *Agouti* locus, which is characterized by obesity, hyperinsulinemia, insulin resistance, hyperglycemia, hyperleptinemia, increased linear growth and yellow coat color. This syndrome is caused by ectopic expression of Agouti in multiple tissues. Mechanisms of Agouti action in obesity seem to involve at least in part by competitive melanocortin antagonism. Both central and peripheral effects have been implicated in Agouti-induced obesity. An agouti related protein (AGRP) has been recently described and shown to be expressed in mice hypothalamus and to act like agouti as a potent antagonist to central MC4-R, suggesting that AGRP is an endogenous MC4-R ligand. Mice lacking MC4-R become hyperphagic and develop obesity, implying that agouti may lead to obesity by interfering with MC4-R signaling in the brain and consequently regulating food intake. Further, food intake was inhibited by intracerebroventricular injection of a potent melanocortin agonist and was reversed by administration of an MC4-R antagonist. A direct cellular action of Agouti includes stimulation of fatty acid and triglyceride synthesis via a Ca^{2+}-dependent mechanism. Agouti and insulin act in an additive manner to increase lipogenesis. This additive effect of agouti and insulin is demonstrated by the

[1] This manuscript has been accepted for publication in similar form with authours Moustaïd-Moussa, N. and Claycombe, K.J. in: Obesity Res.

necessity of insulin in eliciting weight gain in transgenic mice expressing agouti specifically in adipose tissue. This suggests that agouti expression in adipose tissue combined with hyperinsulinemia may lead to increased adiposity. The roles of melanocortin receptors or agouti-specific receptor(s) in agouti-regulation of adipocyte metabolism and other peripheral effects remain to be determined. In conclusion, both central and peripheral actions of agouti contribute to the yellow mouse obesity syndrome and this action involves at least in part by antagonism with melanocortin receptors and/or regulation of intracellular calcium.

B. Introduction

Obesity is a complex phenotype, which involves both genetic and environmental factors. Animal models have been extensively used as a tool to elucidate human obesity genes and mechanisms of human obesity. Several autosomal dominant (yellow) and recessive (obese, diabetes, fatty, tubby, fat) obesity mutations have been described in mice and rats (reviewed in reference No 1). While these mutants have been studied for many decades, the molecular cloning of these genes has been achieved in the last five to six years providing a novel approach to analyze obesity at the molecular level. *Agouti* is the first obesity gene to be cloned (2) and this review will focus on the role of the *Agouti* gene product in the yellow mouse syndrome. Furthermore, human agouti is a closely related homologue of the mouse agouti gene that is 80% identical overall and 87% identical within the carboxyl-terminus (3). Therefore, investigation of mechanisms of agouti-induced obesity may provide insight into human obesity. Interestingly, unlike in mice where agouti is only expressed in skin during hair growth, human agouti is normally expressed in adipose tissue, suggesting a possible direct regulation of agouti on lipid metabolism.

C. Metabolic abnormalities of the yellow mouse

The *agouti* gene is normally involved in coat color regulation in wild type mice. Expression of agouti in the hair follicle during early postnatal development causes a switch from black (eumelanin) to yellow (phaeomelanin) pigment synthesis, resulting in a subapical yellow band on otherwise dark hair (4).

In addition to a characteristic yellow coat color, dominant mutations at the agouti locus such as the lethal yellow (A^y/a) and viable yellow (A^{vy}/a) develop a pleitropic syndrome (4-6) of increased linear growth, increased susceptibility to cancer, insulin resistance and maturity-onset obesity. This obesity is associated with body fat levels that are 35-50 % greater than wild type mice (7) and may be due to elevated hepatic lipogenesis (8), adipocyte hypertrophy rather than hyperplasia (9) and depressed basal lipolysis (4). In these mutants, regulated expression of *Agouti* is disrupted, resulting in ectopic expression in almost all tissues examined throughout the life of the mouse. Both *ob* and *db* mutants exhibit uncontrolled feeding behavior, a positive energy balance and early-onset obesity (10). Conversely, although the yellow agouti mutants tend to consume more food than their lean littermates (4, 8, 11), they are less hyperphagic than ob/ob or db/db mutants suggesting minor alteration in their satiety mechanisms (5). In addition, neither the moderate hyperphagia, nor the decreased thermogenesis (12) can fully account for the yellow mouse obesity (4). Feeding studies have suggested that yellow mice have normal satiety mechanisms but have a stronger motivation to consume food than normal mice (5). In addition, it appears that alterations in the efficiency of food utilization rather than thermogenesis are primarily involved in regulation of body weight in A^{vy}/a mice in response to restricted food intake (reviewed in 4). The above observations are consistent with a possible hypothalamic defect that results in an abnormally higher set point for body weight in yellow obese mice (reviewed in 5) .

Hyperinsulinemia is evident in A^{vy}/a mice at 6 weeks of age and become as high as 20-fold over lean controls by 6 months of age (4). Thus, hyperinsulinemia may increase

nutrient utilization and adipocyte hypertrophy, and contributing to obesity in the yellow mouse (4, 5). In addition, pancreatic β- cell hyperplasia is apparent in A^{vy}/ a males at 21 days of age before any detectable weight gain or changes in insulin or glucagon levels (13). However, the relationships between hyperinsulinemia, insulin resistance and obesity is complex, and it is not plausible to attribute the obesity in the yellow mouse to the hyperinsulinemia alone (4): Hyperglycemia and possibly the moderate hypercorticosteronemia may also contribute to excess triglyceride stores in the obese yellow mouse. Furthermore, several studies have shown that the predisposition of A^{vy}/a mice to become obese is not dependent on pituitary, adrenocortical, or thyroid hormones, since neither hypophysectomy (14), adrenalectomy (15), nor genetic deficiency of growth hormone or thyrotropin (7) prevents the development of obesity in yellow mice. However, pituitary and adrenal hormones appear to be necessary for the complete expression of the yellow mouse syndrome (reviewed in 5). In addition, parabiosis experiments between A^{vy}/a and nonagouti (*a*/*a*) littermates demonstrate that the obese yellow mouse phenotype is not due to circulating Agouti (16). These experiments suggest that agouti modifies adiposity through paracrine actions, consistent with its mechanism of action within the hair follicle.

D. Molecular characterization of agouti and mouse models of agouti-induced obesity

Agouti was the first obesity gene to be cloned (2). Murine agouti mRNA is not expressed in adult tissue (except in testis) but it is expressed in neonatal skin in a manner that correlates with its role in pigmentation. Agouti gene encodes a 131 amino acid

protein with a consensus signal peptide (17). This protein contains four features: 1. An amino-terminal signal peptide, characteristic of secreted proteins, 2. A central region where 16 out of 29 amino acids are basic arginine or lysine residues, 3. A poly-proline stretch that follows the basic region and 4. A cysteine-rich carboxy-terminal domain. This carboxyl domain is as biologically active as the full length protein in vitro (18, 19). An additional feature of the carboxyl terminal region is that the 10 cysteine residues are spaced similarly to the conserved ordering of cysteines in a large group of neurotoxins found in the venom of the primitive hunting spiders and cone snails (20). All 10 cysteines in the agouti protein are disulfide bonded in a pattern (19) that is consistent with the pattern of disulfide bonds in the agatoxins (20). Obese yellow mice exhibit ubiquitous and strong expression of the wild type agouti coding sequences. This ubiquitous expression of agouti results from the deletion of a 170kb fragment that includes the Raly (Ribonucleoprotein Associated with Lethal Yellow) gene located upstream of the agouti coding sequence. Deletion of this fragment leads to *Agouti* gene expression under the control of this ubiquitous Raly promoter in all tissues (21) . Transgenic mice in which the wild-type Agouti cDNA is placed under transcriptional regulation of another ubiquitous promoter (β-actin, BAPa) not only express the agouti mRNA in multiple tissues, but also develop obesity, hyperinsulinemia, hyperglycemia and yellow coat color (22). Agouti was expressed in these mice in all tissue at or above the levels found in A^y mice. Transgenic males became 30 to 40% heavier and females 60-70% heavier than nontransgenic controls (22). Furthermore, the basal core temperature was significantly depressed in transgenic compared to control mice (23), indicating that decreased thermogenesis may contribute to

positive energy balance in these mice. Both male and female transgenic mice became hyperinsulinemic within 12 wk. of age whereas only males developed overt hyperglycemia (22). Insulin to glucose ratios in these transgenic mice were twice that of non-transgenic mice. These results indicate that ectopic expression of Agouti recapitulates the yellow mouse syndrome and demonstrates that ectopic expression of Agouti *per se* is responsible for the disease syndrome associated with dominant Agouti alleles (22). These results suggest that expression of the agouti gene in a novel target tissue(s) triggers the development of obesity in this model. This issue was addressed by generating transgenic mice expressing agouti in specific tissues. Mice specifically overexpressing agouti in skin (24), or adipose tissue (25) do not become obese. However, insulin injections in transgenic mice overexpressing agouti in adipose tissue leads to a moderate weight gain (25).

Mutational analysis of agouti revealed that the same structural features of agouti are generally important for both the production of yellow pigment and the development of obesity (26). Transgenic mice with a mutation in the signal peptide of Agouti do not become yellow or develop obesity while deletion of half of the central basic region of agouti did not significantly impair the development of yellow fur or obesity. However, substitution of individual cysteines with serine residues at some positions in the agouti carboxyl terminus completely eliminated the potential for both yellow pigmentation and obesity (26). Mutations in the carboxyl terminus of the agouti protein decreased agouti inhibition of ligand binding to the melanocortin receptor as well as obesity (18, 26).

E. Role of melanocortin receptors in agouti signaling

The mechanism of Agouti regulation of mouse coat color is due to competitive antagonism of α-melanocyte stimulating hormone (α-MSH) binding (27), resulting in suppression of cAMP production and a shift from eumelanin (black pigment) to phaeomelanin (yellow pigment) production. Accordingly, this mechanism served as a paradigm for investigating Agouti regulation of obesity. Agouti antagonizes the binding of α-MSH to MC1-R (melanocyte melanocortin-1 receptor) and blocks the increase in cAMP, leading to default synthesis of phaeomelanin. In the absence of Agouti, α-MSH binds to MC1-R inducing eumelanin synthesis in melanocytes. This antagonistic action of agouti was demonstrated in human embryonic kidney (HEK 293) cell line transfected with MC1-R (27). In addition, Agouti increased intracellular calcium only in cells transfected with MC1-R or MC3-R receptors but not in non-transfected HEK 293 cells (28).

Although yellow fur generally correlates with obesity, yellow pigment production *per se* is not critical for the development of obesity. A^{vy}/a mice carrying a dominant black mutation (constitutively active MC1-R mutant) have black fur and become obese (29), indicating independence of the two phenotypes from each other and that agouti does not act via MC1-R to induce obesity. Interestingly, Lu et al. (27), demonstrated that agouti also antagonizes another melanocortin receptor (MC4-R) that is widely expressed in brain, particularly regions of the hypothalamus (30) that are directly involved in body weight regulation (5). Targeted disruption of MC4-R recapitulated the yellow mouse syndrome (31): However, the magnitude of hyperinsulinemia, hyperglycemia and hyperphagia were significantly higher than that observed in A^{vy}/a or BAPa transgenic mice (transgenic mice

ectopically expressing agouti under the control of the β –actin promoter) of the same genetic background. This suggests that central effects of agouti via MC4-R may only account for part of the yellow mouse obesity. However, this may be due to differences in MC4-R activity between the MC4-R knockout (loss of MC4-R activity) and the agouti mutant (incomplete inhibition of MC4-R activity by agouti).

While MC4-R is a possible candidate for agouti-induced obesity, MC1-R (MSH receptor) is not likely to be involved based on studies demonstrating that agouti expression in skin results in yellow fur but not alterations in body weight or hyperglycemia (24). MC5-R, a ubiquitously expressed receptor of the same family (32) is not likely a target based on cell based assays demonstrating that agouti does not antagonize this receptor (18, 27). However, MC3-R and MC2-R remain potential targets for Agouti in peripheral tissues. MC3-R (30) is expressed in the limbic system, hypothalamus, placenta, and gut. Agouti is a high affinity antagonist for human (but not rat) MC3-R (18, 27). It is not known whether agouti antagonizes MC2-R (ACTH receptor), although it is well known that functional MC2-R receptors are expressed in adipocytes (33).

The human homologue to mouse agouti, agouti signaling protein (ASIP), is also a competitive antagonist to α-MSH at MC1-R (34). ASIP is a potent inhibitor of human hMC1R, MC2-R and MC4-R and has been suggested to play, like murine agouti, a role in modulating melanocortin receptor signaling in humans (34). Although

linkage analysis failed to find an association between human obesity and the mouse agouti homologous region (or other markers in chromosome 20q), recent studies uncovered new restriction fragment-length polymorphism (RFLP) for MC4-R and MC5-

R (chromosome 18p21.3-q22 and 18p11.2) and reported evidence of linkage/association of these receptors with obesity-related phenotypes in subjects form the Quebec Family Study (QFS) (35).

F. Central effectors involved in agouti action

Agouti induction of obesity is likely to be mediated by both central and peripheral mechanisms, as documented below, via mechanisms that may or may not involve melanocortin receptors (figure 2). Recent studies implicated the hypothalamus, and in particular the arcuate nucleus, in agouti-induced obesity. As discussed in the above paragraph, agouti may in part promote obesity via MC4-R antagonism. This is further supported by studies showing that intracerebroventricular injection of a potent melanocortin agonist (MTII) inhibited feeding in models of hyperphagia including fasted C57BL/6J, ob/ob, A^y/a and mice injected with NPY (36). This group further identified the dorsal medial hypothalamic nucleus as a brain region that is functionally altered by the disruption of melanocortinergic signaling, possibly via elevation of neuropeptide Y (37). NPY was elevated in two genetic obesity models: MC4-R knockout mice and A^y mice.

These data demonstrate that Agouti may promote obesity via antagonism of central MC4-R receptors. A novel protein product of agouti-related transcript (ART) also called agouti related protein (AGRP) has been recently described (38, 39). ART encodes a 132 amino acid protein, which is 25% identical to human agouti with the highest degree of identity in the carboxyl terminus and 9 out of 11 cysteines are conserved (38). The mouse homologue of ART is 81% identical at the amino acid level to human ART and

exhibits the same distribution (39). ART maps to human chromosome 16 and mouse chromosome 8. This protein also acts in vitro as a potent antagonist to human MC3-R and MC4-R but a weaker antagonist to human MC5-R (38, 39). Since ART (but not agouti) is normally expressed in the hypothalamus, this protein is more likely the endogenous central melanocortin receptor antagonist regulates food intake (38). Ubiquitous expression of human AGRP cDNA in transgenic mice caused obesity without altering pigmentation: This obesity resembles that of the MC4-R knockout, indicating that AGRP acts as an antagonist at MC4-R for MSH binding to this receptor. AGRP levels were 8 to 10-fold higher in *ob/ob* and *db/db* mice compared to lean controls, indicating that AGRP is a neuropeptide implicated in the normal control of body weight downstream of leptin signaling (39).

G. Peripheral effectors involved in agouti action

Our recent data support involvement of peripheral tissues, particularly adipose tissue, in the obesity syndrome of the yellow mouse. Boston and Cone have demonstrated that MC2-R (ACTH receptor) and MC5-R are expressed in adipose tissue and 3T3-L1 cells (34). In contrast to central actions of melanocortin ligands discussed above, we have demonstrated that NDP-MSH, a potent α-MSH agonist was unable to reverse obesity and associated metabolic abnormalities in mice overexpressing agouti either by mutation (A^y/a) or transgene introduction (ubiquitous expression of agouti). However, this compound showed effective systemic effects on coat coloration and core temperature (40). In addition, studies using human kidney cells demonstrated that agouti was able to regulate

calcium signaling only in cells transfected with melanocortin receptors (28). Taken together, these studies suggest that agouti-dependent metabolic effects are mediated via melanocortin receptors but not via antagonism of these receptors and that agouti may work via its own specific receptor. This is in contrast to central effects of agouti discussed above implicating melanocortin (MC4-R) antagonism in agouti-induce obesity.

Since the yellow mouse phenotype involves paracrine and not endocrine actions of agouti (16), lipogenic tissues are likely targets for the agouti gene product. Elevated rates of hepatic lipogenesis and increased adipocyte size have been described in A^{vy}/a mice relative to lean controls (4). These reports suggest that elevated rates of lipid synthesis and/or storage in liver and adipose tissue contribute to the obese phenotype of the yellow mouse. Indeed, we have recently demonstrated that agouti induced a 2 to 3- fold increase in *de novo* lipogenic gene, fatty acid synthase (FAS), expression as well as an increase in the triglyceride content of murine 3T3-L1 adipocytes (41). This is consistent with our recent data showing overexpression of FAS in adipose tissue of obese A^{vy} as well as transgenic mice ubiquitously expressing agouti under the control of β-actin promoter, relative to lean, nonagouti controls (23, 41).

Intracellular calcium levels are elevated in obese yellow mouse compared to lean controls (42). In addition, recombinant agouti protein elevates $[Ca^{2+}]_i$ in various cell types including adipocytes (28, 40). Furthermore, we demonstrated that agouti enhances FAS expression and triglyceride storage in 3T3-L1 adipocytes and promotes obesity at least in part through a $[Ca^{2+}]_i$-mediated signaling mechanism (23, 40). Agouti-

stimulation of FAS expression both in vivo ad in vitro was inhibited by Ca^{2+} entry blockade with nitrendipine or nifedipine (23, 41). These findings suggest that perturbations in calcium signaling and calcium homeostasis by the agouti protein may contribute to insulin resistance and obesity of the yellow mouse. These studies indicate that peripheral actions of agouti contribute to yellow mouse obesity; it is possible however these anabolic effects may also limit substrate availability, further contributing to weight gain.

H. Agouti-insulin interaction

Transgenic mice expressing agouti under the control of the adipocyte specific fatty acid binding protein promoter, aP2 (aP2a), expressed very high levels of agouti in brown and white adipose tissue (25). However, these mice do not become obese or hyperinsulenemic, indicating that expression of Agouti in adipocytes alone is not sufficient to induce obesity. Interestingly, when aP2a mice were given daily subcutaneous injections of insulin for one week, the transgenic mice gained significantly more weight (1.7-fold) compared to their non-transgenic littermates (25). These findings suggested that agouti and insulin act together to promote weight gain *in vivo*, possibly via their lipogenic and/or antilipolytic effects (3, 4). Indeed, we have recently confirmed this hypothesis *in vitro* by demonstrating that agouti increases lipogenesis (41) and inhibits lipolysis (42) in adipocytes. Furthermore, consistent with *in vivo* data, agouti and insulin acted in an additive manner to increase fatty acid synthase gene transcription (43). Interestingly, distinct sequences in the FAS promoter mediate agouti and insulin response and current

studies are characterizing these sequences and nuclear adipocyte proteins interacting with these sequences.

In addition to Agouti expression in adipose tissue, we have recently shown that agouti is also expressed in rat pancreatic cell lines (44) and in human pancreas (unpublished data) where agouti increased intracellular calcium and insulin secretion (44). These data indicate that a combination of hyperinsulinemia and normal agouti expression in human adipose tissue may contribute to increased adiposity.

I. Agouti-leptin interaction

Several neuropeptides including α-MSH are derived from the precursor pro-piomelanocortins (POMC) gene. As discussed above, obese yellow mice exhibit defective melanocortin signaling in the brain, due to agouti antagonism at central MC4-R and express high levels of leptin (reviewed in 45). Consequently, recent studies have investigated the role of interaction between leptin and agouti signaling in obesity.

α-MSH may act as an agonist at the MCR-4 to decrease feeding, possibly via interaction with the leptin signaling pathway. In the arcuate nucleus (which also expresses NPY), 30% of the POMC neurons express leptin receptor mRNA long form and the arcuate POMC mRNA is upregulated by leptin (47). In addition, POMC levels were reduced in *db/db* and *ob/ob* compared to lean animals, while leptin injection to *ob/ob* mice but not *db/db* enhances POMC levels to those found in lean controls (47, 48). Thus, it is conceivable that high levels of leptin induce POMC which then leads to increased MC4-R activation via MSH, resulting in decreased food intake. Consistent with high leptin levels

in obese yellow mice compared to lean controls (29), agouti transgenic mice (whether ubiquitously expressing agouti in all tissues or specifically in adipose tissue) exhibited higher levels of leptin compared to non-transgenic controls (40, 50 and Claycombe et al., submitted for publication). Despite these high levels of leptin, this "leptin resistance" is not due to genetic defects blocking leptin action in obese yellow mice. This conclusion was supported by recent studies by Boston et al., (51) that demonstrated that removal of leptin from the obese yellow mice (double mutant for *Agouti* and *ob*) restores complete leptin sensitivity, strongly arguing that animals are leptin resistant as a consequence of desensitization to further leptin action. This suggests that yellow mouse obesity is independent of leptin action. In agreement with these findings, melanocortin ligands were able to regulate food intake in leptin deficient (*ob/ob*) mice (36), suggesting that MCR signaling is intact in these mice and does not require functional leptin to regulate feeding behavior. Accordingly, it is reasonable to propose that increased leptin secretion in obese yellow mice may serve peripherally to limit agouti-induced obesity, while central effects of leptin are attenuated by agouti antagonism of MC4-R. Alternatively, POMC products(s) may serve as integrative link between leptin and central mechanisms regulating food intake (45).

J. Conclusion

Collectively, available data indicate that agouti regulates adiposity via central and peripheral mechanisms (figure 2). Centrally, agouti acts via melanocortin antagonism at MC4-R to regulate food intake. The recent discovery that an agouti related protein,

AGRP is highly expressed in hypothalamus suggests that this protein is a natural melanocortin ligand involved in normal regulation of food intake. Future studies will elucidate the mechanisms of agouti and central melanocortin receptors in obesity and the relationship between agouti and leptin in regulation of food intake. Peripherally, agouti acts as a lipogenic and antilipolytic hormone, stimulating insulin secretion and further promoting triglyceride storage at least in part via a Ca^{2+}-dependent mechanism. Whether melanocortin receptors or agouti specific receptor are involved in this peripheral regulation remain to be elucidated.

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