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Role of Intracellular Free Calcium in the Obesity and Insulin Resistance Associated with Dominant Agouti Mutations

Jung Han Kim
University of Tennessee, Knoxville

To the Graduate Council:

I am submitting herewith a dissertation written by Jung Han Kim entitled "Role of Intracellular Free Calcium in the Obesity and Insulin Resistance Associated with Dominant Agouti Mutations." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Human Ecology.

Michael B. Zemel, Major Professor

We have read this dissertation and recommend its acceptance:

Jay Whelan, Naima Moustaid, Richard P. Woychik

Accepted for the Council:
Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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ROLE OF INTRACELLULAR FREE CALCIUM IN THE OBESITY AND INSULIN RESISTANCE ASSOCIATED WITH DOMINANT AGOUTI MUTATIONS

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

Jung Han Kim
August 1996

DEDICATION

This dissertation is dedicated to my mother, ***Mrs. Kyung Ja Lee***

and to the memory of my father, ***Mr. Hyo Jin Kim***.

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ABSTRACT

Several dominant mutations at the agouti locus in the mouse cause a syndrome of adult-onset obesity, hyperinsulinemia, and insulin resistance. Although ectopic overexpression of the agouti gene is directly responsible for the disease in these mutations, the precise mechanism is unclear. Intracellular Ca^{2+} ($[Ca^{2+}]i$) appears to have a role in mediating insulin signal transduction, and altered handling of $[Ca^{2+}]i$ homeostasis and flux is observed in obese and insulin resistant animals and humans. Data reported here demonstrate that mice carrying the dominant agouti mutation, viable yellow (A^{vy}), exhibit an elevation of $[Ca^{2+}]i$ and Ca^{2+} influx rate in insulin-sensitive type I skeletal muscle. The degree of elevation in $[Ca^{2+}]i$ is highly correlated with the degree of expression of agouti gene and the elevation of body weight. Moreover, recombinant agouti protein directly induced a sustained increase in $[Ca^{2+}]i$ in cultured myocytes and adipocytes; this effect is substantially inhibited by Ca^{2+} channel blockade. Ca^{2+} channel blockade was also effective in reducing fat pad mass and fatty acid synthase (FAS) mRNA levels and activity in adipocytes of transgenic mice expressing the agouti gene in a ubiquitous manner. These results are consistent with previous reports in which recombinant agouti protein directly stimulates FAS mRNA levels and activity and triglyceride content in cultured adipocytes in a Ca^{2+} dependent manner. Accordingly, altered $[Ca^{2+}]i$ metabolism appears to be involved in development of obesity syndrome in A^{vy} mice, and this defect in Ca^{2+} signaling may cause activation of FAS either directly or indirectly and subsequent *de novo* lipogenesis, contributing accumulation of fat depot in these mice.

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INTRODUCTION

INTRODUCTION

Dominant mutations at the murine agouti locus, including viable yellow (A^{vy}), lead to ectopic expression of the agouti gene and result in mice which are characterized by a predominantly yellow coat color and metabolic derangements including obesity, hyperinsulinemia and insulin resistance (1, 2). The mouse agouti gene normally regulates differential pigment production in melanocytes (3). This agouti regulation of pigment production is mediated by competitive antagonism of α-melanocyte-stimulating-hormone binding to its receptor in melanocytes, resulting in suppression of cAMP production and consequent shift from eumelanin (black) to phaeomelanin (yellow) production in melanocytes (4). However, the mechanism linking this pigmentation gene to development of the obesity syndrome has not yet been identified.

Obesity is generally associated with hyperinsulinemia and insulin resistance (5-10). Although the precise mechanism for this insulin resistance is poorly understood, several studies have indicated a potential role for Ca^{2+} in mediating insulin signal transduction (11-19) and that insulin regulates intracellular free Ca^{2+} ($[Ca^{2+}]i$) (20, 21). An optimal range of $[Ca^{2+}]i$ for maximizing insulin action has been demonstrated, such that increasing $[Ca^{2+}]i$ beyond this range results in attenuation of insulin-stimulated glucose uptake in both human and rat adipocytes (21). In support of this concept, obese and insulin resistant patients exhibit higher basal $[Ca^{2+}]i$ in adipocytes than normal individuals, and Ca^{2+} channel blockade decreases $[Ca^{2+}]i$ and improves insulin sensitivity in these subjects (22).

Consequently, this study investigated; (1) whether the dominant agouti mutation, A^{vy}, exhibits altered $[Ca^{2+}]i$ metabolism in insulin sensitive tissue(s); (2) whether

recombinant agouti protein directly influences $[Ca^{2+}]i$ in insulin sensitive cells in culture; (3) whether melanocortin receptors play a role in agouti-mediated Ca^{2+} signaling; (4) whether Ca^{2+} channel antagonism attenuates agouti-induced activation of fat storage and ameliorates insulin resistance in mice overexpressing the agouti gene.

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PART 2

LITERATURE REVIEW

I. PHYSIOLOGICAL OBSERVATIONS IN THE YELLOW MOUSE

The agouti locus is one of several genetic loci that regulate pigmentation in mammals. The agouti locus in chromosome 2 in the mouse controls the alternating production of pigment granules, eumelanin (black) and phaeomelanin (yellow), that are deposited in growing hairs (1). The wild-type agouti mouse produces both eumelanin and phaeomelanin within the same hair bulb melanocytes, in a sequential manner. The sequence of pigmentation during hair growth is such that eumelanin is deposited in the tip of the newly forming hair and then phaeomelanin is deposited for a short time, during the period of time when the hair is growing most rapidly (2, 3). The melanocytes then switch back to eumelanin production for the remainder of the period of hair formation, resulting in agouti coloration of black with a subapical band of yellow. Nineteen different alleles have been identified at the agouti locus, resulting in an intricate dominance hierarchy in which alleles associated with phaeomelanin production are generally dominant over alleles associated with eumelanin production (4).

Lethal yellow (A^y) is the most dominant of the agouti locus alleles, and results in a uniformly yellow coat. The second most dominant allele in the agouti hierarchy is called viable yellow (A^{vy}); interestingly, this mutant allele results in extremely variable coat colors ranging from totally yellow through all degrees of intermixtures of yellow, black, and agouti to totally agouti. Next to yellow fur, these dominant agouti mutations are particularly interesting because they result in mice that exhibit obesity, hyperinsulinemia, and non-insulin-dependent diabetes.

A. Obesity

1. Hyperphasia and efficiency of food utilization

Between 3 and 4 weeks of age, yellow mice start showing weight divergence with their non-yellow littermates; they continue to gain weight at a more rapid rate, reaching a maximum plateau at about the 7 months of age (5, 6). Young yellow obese mice are hyperphagic, while after 40 days of age yellow mice significantly exceeded their black littermates in weight gain with only moderate increase in food consumption, indicating a greater metabolic efficiency in converting food energy to body fat (7, 8). Accordingly, unlike mice with hypothalamic obesity, hyperphasia does not appear to be the major mechanism of obesity in the dominant agouti mutations.

2. Lipogenesis, triglyceride content, and lipolysis

Yellow mice exhibit characteristics of maturity-onset type obesity (9). Early in life, they do not have higher lipogenesis rates and have only a moderate increase in carcass and liver triglyceride content (9). However, when they reach maturity, their lipogenesis rate does not demonstrate the normal maturity-related decrease in lipogenesis (9). This is in contrast to other obese mouse models, such as obese (*ob/ob*) and diabetes (*db/db*) that have characteristics of juvenile type obesity, in which higher lipogenesis rates and a greater accumulation of triglyceride occur when they are young (9). Indeed, lipogenesis at maturity is normal in *ob/ob* and *db/db* mice (9). Furthermore, hepatic lipogenesis in A^{vy}/a mice is much lower than that of *ob/ob* and *db/db* mice at corresponding ages (9). The total triglyceride content in the mature male yellow mice is increased approximately 4-fold

(to approximately 25 % of body weight) compared with black controls, while mature male *ob/ob* and *db/db* mice have 40 % and 50 % body fat, respectively (9).

Adipocyte number in adult yellow mice is not significantly different from control values, although the adipocytes are significantly larger cells than in lean controls (10). The enlargement of these adipocytes is a response to the internal milieu, since transplantation of adipose tissue from fat (A^y/a) to lean (a/a) mice or vice versa is followed by acquisition of anatomic characteristics similar to the host (11). In contrast, *ob/ob* mice have both a significantly larger number and size of adipocytes than controls, proposing a classification for genetic obesity based primarily upon the cellular characteristics of the adipose depots (10).

With regard to lipolysis, the basal and agonist stimulated lipolytic rate of adipose tissue isolated from yellow A^{vy}/a mice is approximately 50 % lower than that of black a/a mice (12). As in other obese animals, including *ob/ob* and *db/db* mice, however, the lipolytic response of A^{vy}/a adipose tissue to dibutyryl cyclic AMP is normal, suggesting reduced cAMP signaling mechanisms in adipocytes from these mice (12, 13).

3. Effect of diet and activity

Yellow mice are sensitive to dietary fat content, with weight gain resulting primarily from an increase in energy efficiency (8). Fenton et al. (14) found that feeding a high fat diet caused obesity even in highly inbred yellow mice that did not become markedly overweight in standard conditions. Similarly, Carpenter et al. (15) found that yellow mice gained more weight on high-fat diets than on high-carbohydrate or high-

protein diets. Exercise in squirrel-type cages significantly reduced body weight in yellow adult mice without a significant difference in non-obese mice (15).

4. Thermogenesis

Increased efficiency of energy utilization may result from a defect in the regulation of thermogenesis (16-18). There is evidence of disturbed temperature regulation in A^{vy} obese yellow mice; at low environmental temperatures, the mean rectal temperature of obese mice continued to fall during a one-hour exposure, while that of normal mice fell only during the first 10 minutes and plateau (19). Thus, A^{vy} mice exhibit a clear defect in adaptive thermogenesis. However, their hypothermia is not sufficient to account for their being overweight, as their weight loss induced by food restriction does not result in increased thermogenesis (20).

5. Endocrine status

Early work by Weitze, in 1940, first showed evidence for hormonal involvement in the development of obesity in yellow mice (21). Yellow mice parabiosed with non-yellow mice did not become obese, suggesting that adiposity in yellow mice is caused by a hormonal disorder (21). However, Wolff (22) could not confirm the findings, and instead noted that parabiosis of yellow and black littermates for six months failed to affect body weight or composition in either group.

Conflicting results have been reported after hypophysectomy. At 45-50 days of age, hypophysectomy decreased weight gain both in yellow (A^y/a) and black (a/a) mice compared to sham-hypophysectomized mice (23). However, hypophysectomized yellow (A^y/a) mice still gained more weight than hypophysectomized black (a/a) siblings and

exhibited an excessive accumulation of fat, thereby demonstrating that the pituitary mediated regulatory pathways are not primarily involved in the mechanism of obesity in yellow mice (23). Similarly, Salem et al. (24) found that hypophysectomized yellow (A^{vy}/A) mice still showed more than a 4-fold increase in parametrial fat pad compared to non-operated agouti (A/A) controls. However, they also observed a drastic decrease in body weight and insulin resistance in hypophysectomized yellow mice, suggesting a potential role for the pituitary gland in the maintenance of obesity in yellow mice. However, hypophysectomized control mice were not studied for comparison.

The obesity of yellow mice does not appear to be due to deficiencies of growth hormone or thyrotropin. When the yellow obese mice gene (A^y) is transferred to the dwarf mouse (dw/dw), which congenitally lacks growth hormone, the yellow dwarf ($A^y/a\ dw/dw$) mice still develop obesity to become fat little mice and their growth rate responded to growth hormone administration to a similar degree as the black dwarf ($a/a\ dw/dw$) mice, implying that growth hormone is not necessary for the expression of the obesity (25).

The role of adrenal hormones in the development of obesity in yellow mice is also controversial. It has been demonstrated that in yellow A^{vy}/a or A^y/a mice, plasma corticosterone levels were similar with those in black (a/a) mice (26), although corticosterone levels were elevated in A^{vy}/a mice in another study (27). Adrenalectomy produced a 30- 33 % reduction in fat deposition in both yellow (A^y/a) and black (a/a) mice, but adrenalectomized yellow mice still had a larger percent of body lipid composition and were fatter than adrenalectomized black mice seven months after surgery (28). Shimizu et al. (27) demonstrated that adrenalectomy significantly reduced the

weight gain in both yellow obese (A^{y}/a) and lean black (a/a) mice thirty-two days after the operation; this effect was especially prominent in the adrenalectomized yellow mice, where the gain in body weight decreased to almost the same level as in the sham-operated controls.

An increase in circulating insulin levels is exhibited in yellow obese $A^{y}/-$ and $A^{vy}/-$ mice at approximately 6 weeks of age, when the animals become overtly obese (29); *db/db* mice are hyperinsulinemic at 10 days of age (30) and hyperinsulinemia in *ob/ob* mice is detectable by 4 weeks of age (31). At 6 months of age, when the obesity of yellow A^{vy}/a mice peaks, plasma insulin level is more than 20 fold that of black a/a in both male and female (32). Hyperinsulinemia may be essential for the expression of the obesity since pseudoagouti (A^{vy}/a), which are phenotypically agouti, mice have normal plasma insulin and fail to gain weight, whereas phenotypically yellow (A^{vy}/a) mice have high plasma insulin and become obese (33). However, the role of insulin in obesity in yellow $A^{vy}/-$ mice is not clear (34).

B. Carbohydrate metabolism

1. Hyperglycemia

Fed yellow obese mice have higher blood glucose concentrations than non-obese controls (24, 35), whereas fasted yellow obese have normal blood glucose values (24, 16). Blood glucose changes in response to cortisone, adrenocorticotropic hormone, growth hormone, and glucagon is exaggerated in yellow obese mice when compared to normal controls (16). Plasma glucose level of yellow (A^{vy}/A) exhibit a sexual dimorphism in that males are hyperglycemic and most females are either normoglycemic (29) or only mildly

hyperglycemic (32). Indeed, this gender difference was also exhibited in plasma insulin concentration; compared to that in the yellow males, the plasma insulin concentration in yellow females began to increase above the normal agouti values at a later time and to a lesser extent (29). Further, glucose tolerance of A^{vy}/a males is more impaired than that of female A^{vy}/a mice (29). However, administration of dexamethasone overwhelmed the protective mechanism against hyperglycemia in female viable yellow mice and induced hyperglycemia (36).

Several glucose tolerance determinations in A^{vy}/A mice of various ages (3-8 months) have shown that tolerance to glucose remains impaired in spite of the very high plasma insulin values (400-500 μU/mL), an indication of an insulin resistant condition (29). In addition, adipose tissue from A^{vy}/A mice required more insulin to stimulate glucose oxidation than in A/a mice, verifying that yellow A^{vy}/A mice are less sensitive and less responsive to insulin than A/a mice (29).

Ciglitazone (5-[4(1-methylcyclohexylmethoxy)benzyl]thiazolidine-2,4-dione), a compound known to improve insulin sensitivity, improved glucose tolerance in the presence of exogenous insulin in yellow A^{vy}/a mice (32). Ciglitazone also prevented and reversed dexamethasone-induced hyperglycemia in female viable yellow mice (36).

2. Pancreatic islet hyperplasia and hypertrophy

Hypertrophy and hyperplasia of the islets of Langerhans are consistent features observed in pancreata of yellow obese mice (37). The pancreatic islets of the 6-month-old male yellow mice were characterized by considerable capillary dilation, with deregulation and increased nuclear size of the β-cells and a concomitant increase in total islet volume

(35). This increased total volume of the hyperplastic islet tissue in the yellow obese mice may reflect the adaptation of the β-cells to increased insulin demand (35). It has been also reported that increased β-cell proliferation occurs between 14 and 21 days of age in yellow A^{vy}/A, prior to any detectable hyperinsulinemia, indicating morphological changes precede biochemical changes in pancreatic islets (38).

II. MOLECULAR BASIS OF GENETIC OBESITY IN RODENTS

A. Obese mouse (*ob/ob*)

The *obese* (*ob*) gene in the mouse chromosome 6 is specifically expressed in white adipose tissue and encodes a 4.5-kb mRNA with a 167 amino acid open reading frame. The predicted polypeptide is largely hydrophilic and contains a putative N-terminal signal sequence (39). The *ob* mutation is autosomal recessive and results in mice that exhibit a juvenile-onset obesity and type II diabetes (reviewed in refs. 40, 41). The mutant allele in C57BL/6J *ob/ob* mice causes a nonsense mutation resulting from a single base substitution (C $\rightarrow$ T) in coding sequences that results in a change of an arginine to a stop condon (39). Northern analysis reveals that two different *ob* mutant strains exhibit a different level of *ob* mRNA expression in adipose tissue; C57BL/6J *ob/ob* mice exhibit overexpression while SM/Ckc-+ ^{Dac} ob^{2J}/ob^{2J} mice do not express. However, the molecular mechanisms leading the altered expression in those mutations are under investigation (39).

The working hypothesis for *obese* action is that a circulating protein-based signal, generated in adipose tissue, acts on central neuronal networks and plays a role in the regulation of feeding behavior and energy balance.

The human homologue of the mouse *ob* gene has an 84 % amino acid sequence homology with extensive signal sequence identity in the N-terminus of the mature protein (39).

Several studies have characterized the *ob* gene product, which has been named leptin, as a circulating signal for the regulation of body weight. Peripheral or central supply of the mouse recombinant leptin reduces food intakes, body weight, serum glucose

and insulin, and increases metabolic rate and body temperature in *ob/ob* mice (42-44). Human recombinant leptin also reduces body weight, body fat, and plasma glucose in *ob/ob* mice (43).

The mouse OB protein is found from normal mouse plasma, but not in plasma from C57BL/6J *ob/ob* mice; similarly, the human OB protein is present in plasma from normal humans, suggesting that the OB protein serves an endocrine function to regulate body fat store (43). In contrast, obese humans and rodents (other than *ob/ob* mice) are not defective in their ability to produce leptin mRNA or protein and generally produce higher levels than lean individuals (45).

The receptor for leptin, OB-R, has recently been characterized and appears to be a single membrane-spanning receptor with 816 amino acids extracellular domain followed by 23 amino acids transmembrane domain and either 34 or 302 amino acids cytoplasmic domain, via alternative splicing (46, 47). These splice variants have been named as OB-R short form or long form transcripts, respectively (47). Transcripts of the two forms are identical until the fifth codon 5' of the stop codon of the short form, at which point they diverge completely, suggestive of alternative splicing (47).

OB-R is expressed in choroid plexus and several other tissues including hypothalamus. Genetic mapping of the gene encoding OB-R shows that it is within the 5.1 cM interval of mouse chromosome 4 that contains the *db* locus (46).

Human OB-R homologue has a 78 % amino acid sequence homology compared with mouse OB-R short form, and encodes 303 amino acid intracellular domain (46). The

mouse OB-R long form and its human homologue share 71 % identity within their intracellular domains (47).

B. Diabetes mouse (*db/db*)

Diabetes (*db*) is an autosomal recessive mutation located on mouse chromosome 4 that arose spontaneously in the C57BL/KsJ (BL/Ks) strain (48). Mice homozygous for the *db* mutation are characterized by hyperphagia, early-onset obesity, hyperglycemia, hyperinsulinemia, and degenerative changes in the islets of Langerhans, the severity of which depends upon the inbred strain on which the mutant allele has been bred (reviewed in ref. 41).

The *db* mutation results in an obese phenotype similar, if not identical, to that seen in *ob/ob* mice, and *db/db* mice have been hypothesized to be resistant to *ob* gene product. When an *ob/ob* mouse is parabiotically joined to its lean littermate the obese mouse exhibited normalization of body weight; in contrast, when a *db/db* mouse is parabiosed to a its lean littermate the obese mouse did not exhibit weight loss (49). When an *ob/ob* mouse is parabiosed to a *db/db* mouse, the body weight of the *ob/ob* mouse is reduced, suggesting the *db/db* mouse secretes a satiety signal to which it cannot respond but that the *ob/ob* mouse does not produce but can respond to (49).

High levels of the OB protein are present in plasma from *db/db* mice. Exogenous administration, either peripherally or centrally, of the mouse recombinant OB protein fails to reduce body weight and food intake in *db/db* mice (43). This again suggests that the genetic defect in *db/db* mice renders them unable to appropriately respond to OB protein,

perhaps because of a defect in the OB receptor or an postreceptor OB signal transduction (43, 44).

Molecular analysis has revealed that *db/db* mice have a 106 nt insertion at the point where the long and short forms of OB-R diverge. The insertion sequence includes sequence encoding the last five amino acids, stop codon, and the first 88 bp of the 3′ untranslated region (UTR) of the short form of OB-R. Further, a genetic point mutation (G→T) is detected 2 nt downstream of the 106 nt insertion at the corresponding region of *db/db* genomic DNA and the transcript then continues as observed for the wild-type long form transcript of OB-R (47). This G→T change creates a new splice donor site, as it is very similar to the consensus sequence (47). Therefore, mutant transcripts of OB-R are produced in *db/db* mice when the common upstream donor site is exclusively spliced to the short form acceptor and the new donor is spliced to the long form acceptor. The net result is two mRNAs encoding identical OB-R proteins with short intracellular domains but with different 3′ UTR, and fails to encode the mature long intracellular domain. This mutation would only affect the intracellular domain of OB-R and thereby radiolabeled OB protein binds as well to the choroid plexus of *db/db* mice as to wild-type mice (46).

This characterization of a mutation in the gene encoding the OB-R protein in *db/db* mice strongly supports the hypothesis that *db* encodes the leptin receptor. Furthermore, genetic mapping and genomic analysis reveals that mouse *db* and the gene encoding the OB-R are the same gene (50). Accordingly, it is predicted that long intracellular domain of OB-R is crucial for initiating intracellular signal transduction of

regulatory pathway for body fat, and as a corollary, the inability to produce this form of OB-R leads to the severe obese phenotype found in *db/db* mice.

C. Fatty rat (*fa/fa*)

The mutation *fatty* (*fa*) first appeared in a cross between Sherman and Merck stock M rats and causes juvenile-onset obesity, hyperphasia, hyperlipemia, and hyperinsulinemia (40, 51). Hyperglycemia is not generally found in *fatty* rats (52).

The *fa* gene maps to rat chromosome 5 in a region of conserved synteny with the mouse chromosome 4 region containing *db,* suggesting that *fa* and *db* are mutations in homologous genes (53). Comparison of genomic DNA from Zucker *fa/fa* rats and +/+ rats on Southern blots probed with mouse OB-R cDNA reveals different hybridization patterns (50). The restriction fragment length variations (RFLV) patterns of the *fa/fa* genomic DNA suggest that the *fa* phenotype is the result of a small insertion or deletion in the OB-R gene (50). On the basis of mapping data and mutation analysis, it has been concluded that *db*, *fa*, and *Obr* are the same gene (50). However, the precise mechanisms by which the mutation produces the apparent loss of function of OB-R remain to be defined.

The obesity of *fatty* rat appears to be transmitted as an autosomal both codominant and recessive trait. An anonymous human DNA probe (VC85) that is tightly linked to the *fa* locus on rat chromosome 5 can detect restriction fragment length polymorphism (RFLP) of Zucker and Brown Norway DNA. Therefore, it is possible with the VC85 probe to genotype segregating *fa* in the progeny of Zucker and Brown Norway intercross (BNZ), although it can not detect the genotype within the Zucker rat strain (54). In F_2

progeny of Zucker and BNZ F_1-*fa*/ + intercross, at 7 days of age, both body weight and fat pad weight are linearly related to the number of copies of *fa* inherited, suggesting that effects of *fa* on body weight and fat pad weight appear codominant rather than recessive at this age (55). At 14 days of age, body weight and fat pad weight clearly increase in the animals carrying two copies of *fa*, but there are without difference between those carrying zero copies and those carrying one copy of *fa*, suggesting that the recessive aspects of the obese phenotype caused by *fa* appear by this age (55).

III. MOLECULAR CHARACTERISTICS OF THE AGOUTI GENE

A. The mouse agouti gene and its expression pattern

The agouti gene in mouse chromosome 2 encodes a 131-amino acid protein containing a consensus signal sequence, a highly basic central region, and a cysteine-rich carboxyl terminal region (56, 57). The agouti gene has three coding exons that are alternatively spliced to two different sets of 5' untranslated exons, resulting in four types of mature 0.7- to 0.8-kb transcripts with identical coding regions. One set is expressed only in the ventrum; the other set in hair-cycle specific and is expressed only during hair growth (56-58). There are two wild-type alleles in the agouti locus, *agouti* (*A*) and *light-bellied agouti* (A^w) (60), although recent studies suggest that the *A* allele may have arisen through mutation of A^w, making A^w the true wild-type allele (58, 59). The *A* mice have agouti hairs all over their body resulting from sole expression of the hair-cycle-specific transcripts, while the A^w mice have an agouti dorsum with a yellow ventrum resulting from a pulse of agouti expression during the hair-growth cycle in the dorsum and continuous expression during the hair-growth cycle in the ventrum (58, 59).

B. Molecular nature of the agouti dominant mutations

Several studies have shown that yellow obese mice result when the agouti gene is ectopically overexpressed (58, 59, 60-65). In fact, the dominant agouti mutations including A^y and A^{vy} are promoter mutations that result from agouti coding exons being placed under the control of ubiquitous promoters. None of the dominant mutations identified to date alter the coding region of the agouti gene.

1. Lethal yellow (A^y) mutation

Lethal yellow (A^y) is a recessive lethal and is associated with a number of dominant pleiotropic effects in the heterozygous condition.

A^y/- mice express size-altered agouti mRNA in all adult tissues examined while wild-type *A/a* mice express agouti mRNA only in the skin during the time of phaeomelanin synthesis (61, 62). A^y-specific agouti mRNA corresponds to the 5' untranslated region of a gene called *Raly*, a ubiquitously expressed heterogeneous nuclear ribonucleoprotein (*hnRNP*) gene, and entire coding portion of the agouti gene (61, 62). This chimeric *Raly*/agouti transcript results from a 170-kb deletion that removes all but the promoter and noncoding first exon of *Raly*, which lies in the same transcriptional orientation as agouti and maps 280 kb proximal to the 3' end of the agouti gene (63). A proposed model to account for the production of the altered-sized *Raly*/agouti chimeric transcripts that are expressed from the A^y allele is that transcription initiates at the *Raly* promoter and proceeds through the first *Raly* exon; however, because the remainder of the gene is deleted, transcription proceeds into the remaining intergenic DNA and through the downstream exons of the agouti gene. The resulting novel primary transcript could be spliced in a manner whereby the splice donor associated with the first exon of *Raly* connects to the available splice acceptors in the downstream exons of the agouti gene, resulting in overexpression of agouti coding region in a ubiquitous manner under the control of *Raly* promoter.

Because *Raly* has the potential to encode a novel RNA-binding protein that is normally expressed in the preimplantation embryo and throughout development, it may

have a critical role in development. Accordingly, the embryonic lethality of the homozygous A^{y} mutation may result from the lack of *Raly* gene expression in the early embryo.

2. Viable yellow (A^{vy}) mutation

Unlike A^{y}, homozygotes of *viable yellow* (A^{vy}) allele is viable, as the name implies. A^{vy}/- mice express agouti mRNA indistinguishable in size in every tissue of the body rather than just in the skin and testis. Molecular cloning studies indicate that A^{vy}-specific RNA is chimeric containing a foreign 5' sequence with the normal agouti coding sequences (64). The foreign 5' sequence in the A^{vy}- specific RNA does not contain an open reading frame with an ATG predicted to initiate translation and is instead derived from an intracisternal A particle (IAP) element which is inserted 100 kb upstream of agouti coding sequences (64). IAP elements are defective retroviruses encoded by a family of endogenous proviral sequences present at about 1000 copies per haploid genome in *Mus musculus* (66). IAP elements are expressed widely during early embryonic development but only in restricted tissues in the adult animal (66), and transpositions to new places in the genome can activate or inactivate the expression of adjacent genes. Factors that control IAP transposition are not well understood. The IAP associated with the A^{vy} allele appears to activate agouti expression by initiating transcription from a cryptic promoter found within the IAP long terminal repeats (LTR) which are met at the termini of the integrated element and carry a promoter and enhancer that can act on cellular as well as viral sequences (64, 65).

The A^{vy} allele is of special interest because the expressivity of the phenotype can vary from one to another, even among siblings of a single litter. A^{vy}/- mice exhibit a broad spectrum of coat colors that range from solid yellow, to yellow with varying amounts of agouti mottling, all the way to a coat color that has been referred to as pseudoagouti, which is similar to wild-type agouti pigmentation. Similar to the coat color variations, the degree of obesity is also heterogeneous. The expressivity of this mutant phenotype is related to the degree of expression of agouti; mice with a solid yellow coat ectopically express agouti mRNA at high levels and exhibit marked obesity, whereas pseudoagouti mice express agouti mRNA at a very low level and remain lean.

Although the precise mechanism for the variegated expression is not clear, this differential expressivity of the A^{vy} allele appears to be correlated with the methylation status of the inserted IAP 5′ LTR. This mechanism is deduced from molecular analysis of a new dominant agouti allele (A^{iapy}) which exhibits very similar genetic and phenotypic characteristics as A^{vy} (65). The regulatory region of the IAP 5′ LTR in pseudoagouti mice carrying A^{iapy} is almost completely methylated, but is hypomethylated in solid yellow mice carrying A^{iapy}. Further, it has been known that the expression of IAP proviral elements is inversely correlated with the methylation state of their 5′ LTRs (67).

3. Transgenic mouse assay

Even though several studies have shown that the action of an ectopic agouti protein is responsible for the obesity, diabetes, and other dominant pleiotropic effects in the dominant agouti mutant mice, the nature of the structural changes introduced in the agouti locus in these mutations, including a 170-kb deletion of 5' flanking DNA and the

insertion of retrotransposable elements within the locus, makes it unclear to unequivocally conclude that the ubiquitous expression of agouti solely causes the pleiotropic phenotypes in these mutations. Transgenic mice in which the expression of a wild-type agouti cDNA is under the influence of ubiquitously expressed promoters, such as human β-actin or mouse phosphoglycerate kinase (PGK) promoter, not only express agouti in a ubiquitous manner but also develop obesity, hyperinsulinemia, and insulin resistance, as well as yellow fur (68, 69). The average weight of transgenic mice derived by β-actin promoter and PGK promoter first become significantly greater than the control by about 4-6 weeks of age and 8-14 weeks of age, respectively (68). By 20 weeks of age, transgenic mice develop significantly higher level of plasma insulin and only male transgenic mice exhibit hyperglycemia (68). Moreover, transgenic mice carrying transgene of β-actin promoter/ agouti cDNA containing a point mutation in the agouti coding region do not exhibit transgene-induced yellow fur coloration, even though transgene expression has been detected at the RNA level in the skin (69). These transgenic models now demonstrate that ectopic overexpression *per se* of the agouti gene is responsible for the phenotypes exhibited by mice carrying dominant agouti mutations.

C. Human homologue of the mouse agouti gene

The human homologue of the mouse agouti gene has been cloned and mapped to human chromosome 20 (70, 71). The human agouti gene has been shown to influence coat color in transgenic mice (71). Sequence analysis reveals that the coding region of the human agouti gene is 85% identical to the mouse gene at the nucleotide level and 80% identical at the amino acid level (70, 71). Like the mouse protein, the amino terminus of

the putative human protein is indicative of the features of a signal peptide (70, 71). The human agouti gene is expressed in adipose tissue, heart, ovary, and testis and to a lesser extent in foreskin, kidney, and liver (70, 71). This presence of agouti transcripts in human tissues may speculate a role for the agouti gene in skin pigmentation, cardiovascular function, energy metabolism, and fertility (72).

IV. MODELS FOR AGOUTI ACTION IN PIGMENTATION

In mammalian pigmentation, tyrosinase is the rate-limiting enzyme involved in synthesis of both eumelanin and phaeomelanin. Decreased tyrosinase activity results in phaeomelanin synthesis, while high tyrosinase activity results in eumelanin production in the melanocyte (reviewed in ref. 73). Tyrosinase activity in melanocytes is regulated by α-melanocyte stimulating hormone (α-MSH). α-MSH stimulates melanin formation in melanocytes by binding to a membrane receptor and activating adenylate cyclase via a G protein-coupled pathway (74). α-MSH induces an increase in the transcription of the tyrosinase gene (75, 76) and activity (77, 78), and thereby induces eumelanin synthesis (79, 80). The relative amounts of eumelanin and phaeomelanin in the melanocyte are controlled primarily by two loci in mammals, *extension* which encodes α-MSH receptor (81) and *agouti*. In contrast to agouti, dominant extension mutation exhibits increased eumelanin pigment, resulting in black mice, while recessive mutation blocks eumelanin synthesis resulting in yellow mice (82).

Previous experiments suggested that injection of the α-MSH promoted black pigment synthesis after injection into dominant agouti mutants, but not in yellow recessive extension mutants, suggesting that agouti is an antagonist of α-MSH which prevents binding or signaling and result in yellow pigment (1). It has been reported that agouti protein is a competitive antagonist of α-MSH, inhibiting α-MSH binding to its receptor and thereby inhibiting α-MSH-induced cAMP (83). The highly cysteine-rich C-terminal domain of agouti protein retains the bioactive characteristic for α-MSH antagonism (84). Therefore, the predominant yellow fur in dominant agouti mutations appears to be through

antagonism of the α-MSH receptor, thereby preventing the elevation of intracellular cAMP and eumelanin production.

V. OBESITY-DIABETES SYNDROME AND INTRACELLULAR CALCIUM

A. Insulin resistance in obesity-diabetes syndrome

Obesity and non-insulin-dependent diabetes mellitus (NIDDM) often coexist and are frequently complicated by insulin resistance (85, 86). In the U. S., 80% of NIDDM patients are obese and about 20% of obese individuals are diabetic (87). Insulin resistance is defined as a subnormal biologic response to a given concentration of insulin. Although insulin's effects are pleiotropic, the term "insulin resistance" typically refers to the actions of insulin on glucose homeostasis. Obesity and NIDDM is also characterized by hyperinsulinemia, secondary to insulin resistance (88). Although fasting insulin levels do not directly measure insulin resistance, there is evidence that the rank order of basal and stimulated insulin levels correlate highly with the reciprocal of tissue insulin sensitivity (89). In addition, several epidemiological studies have found higher basal insulin levels and reduced tissue insulin sensitivity in obese subjects compared with lean subjects (90-92).

B. Insulin signaling

In mammals, insulin is the principal hormone controlling blood glucose and acts by stimulating glucose influx and metabolism in muscle and adipocytes, and inhibiting gluconeogenesis by the liver. In addition, insulin modifies the expression or activity of a variety of enzymes and transport systems in nearly all cells. Insulin's diverse actions are initiated by interaction with a specific receptor, a transmembrane glycoprotein with intrinsic protein tyrosine kinase activity.

The insulin receptor is present in virtually all vertebrate tissues, although the concentration varies from as few as 40 receptors on circulating erythrocytes to more than 200,000 receptors on adipocytes and hepatocytes (93). The human insulin receptor gene is located on the short arm of human chromosome 19, is more than 150 kb in length and contains 22 exons which encode a 4.2-kb cDNA (94). The insulin receptor is composed of two α-subunits that are each linked to two β-subunits and to each other by disulfide bonds. Both subunits are derived from a single proreceptor by proteolytic processing at a cleavage site consisting of four basic amino acids. There is one site of alternative splicing surrounding exon 11, which results in two receptor isoforms differing by 12 amino acids near the COOH terminus of the α-subunit. In the mature heterotetramer ($\alpha_2\beta_2$) receptor, the α-subunits are located entirely outside of the cell and contain the insulin binding site(s), whereas the β-subunits span the plasma membrane and the intracellular portion of the β-subunits contain the insulin-regulated tyrosine protein kinase. After insulin binding, specific tyrosine residues of the β-subunits are rapidly phosphorylated, and the tyrosine kinase intrinsic to this region is activated (95, 96). The immediate consequences of the activation of the tyrosine kinase activity of the insulin receptor remain poorly defined.

In addition to signal transduction, the insulin receptor mediates internalization of insulin. Endocytosis of the insulin-receptor complex leads to insulin degradation, while most of the unoccupied receptors recycle to the plasma membrane. After prolonged insulin stimulation, the receptor itself is degraded, resulting in receptor down-regulation and attenuation of the insulin signal (97, 98). Dephosphorylation of the insulin receptor by tyrosine phosphatases is one likely mechanism for termination of the signal (99).

However, the identity and regulation of specific phosphatases that act on the insulin receptor are not known. Substantial evidence points to a role of serine/threonine phosphorylation of the insulin receptor in inhibitory regulation of insulin signaling. Several serine/ threonine residues have been identified , in C-terminal and juxtamembrane regions of the insulin receptor, which become phosphorylated in response to activation of protein kinase C or the action of insulin itself (100, 101).

C. A potential network of insulin signaling pathways mediated through activation of the insulin receptor kinase and phosphorylation of IRS-1 or Shc

Despite an explosion of information concerning intermediates of intracellular signaling pathways utilized by receptor tyrosine kinases, understanding of the mechanism of cellular insulin action remains poor. The proteins classically regulated by insulin (e.g. glycogen synthase and glucose transporters) are not functionally activated by tyrosyl phosphorylation, while many cellular actions of insulin are regulated by phosphorylation or dephosphorylation of serine/threonine residues.

Accordingly, one hypothesis is that insulin action involves a phosphorylation cascade, with the receptor at one end and enzymes that undergo increased or decrease serine/threonine phosphorylation at the other. In this hypothesis, the first intermediate in the signal transduction pathway must be a protein phosphorylated on tyrosine. With using anti-phosphotyrosine antibodies, insulin receptor substrate-1 (IRS-1) was initially detected in insulin-stimulated Fao hepatoma cells, and originally termed pp185 based on its migration during SDS-PAGE (102). IRS-1 is widely distributed in tissues and contains 21

potential tyrosine phosphorylation sites. At least 8 tyrosines in IRS-1 undergo phosphorylation by the activated insulin receptor (93, 103).

To propagate the signal, IRS-1 binds several proteins containing Src homology 2 domains (SH2 proteins) through its multiple tyrosine phosphorylation sites. The SH2 proteins associated with IRS-1 include phosphatidylinositol (PI) 3′-kinase, SH-PTP2 (Syp), a protein tyrosine phosphatase, and GRB-2, a small cytoplasmic protein that acts as adapter molecule linking the guanine nucleotide exchange factor for $p21^{ras}$, termed mSOS (homologous to the *Drosophila* protein, son-of-sevenless (Sos)) to tyrosyl phosphoproteins (103). The binding of GBR-2/mSOS to IRS-1 may mediate the insulin stimulation of $p21^{ras}$. However, Ras is also stimulated by insulin through insulin-stimulated tyrosine phosphorylation of Shc (104) or the direct binding of $p21^{ras}$ to the insulin receptor (105). Ras has been shown to bind directly Raf-1 serine/threonine kinase, which in turn activates MAP (microtubule-associated protein) kinase by phosphorylation and activation of the MAP kinase kinase (106).

There is an evidence that PI 3′-kinase plays an essential role in insulin signaling to glucose transport, glycogen synthesis and inhibition of lipolysis. In particular, specific inhibitors of PI 3′-kinase block the effects of insulin on these processes (107-109).

Insulin appears to stimulate $p90^{rsk}$ through the MAP kinase cascade and thereby phosphorylate and activate the glycogen-associated protein phosphatase-1, which places stimulation of glycogen synthase (93). MAP kinase may play a role in effects of insulin on gene transcription, but not a major pathway regulating insulin-stimulated glucose transport and glucose transport-4 translocation (110).

The importance of IRS-1 in insulin signaling was confirmed by use of antisense, transfection and microinjection strategies (111-113). However, transgenic mice which lack IRS-1, as a result of targeted gene disruption, display a phenotype much less severe than would be expected if IRS-1 were essential for all aspects of signaling by insulin (114, 115). These mice are modestly insulin resistant and growth-retarded, but otherwise survive remarkably well. Thus it appears that, although IRS-1 plays an important role in insulin signaling, there must be alternative mechanisms for activating pathways essential to insulin's effects on cell growth and metabolism.

Most recently, it has been proposed that there exists another member of the IRS-1 family, p190/IRS-2, which plays a role similar to IRS-1 (115, 116). As PI 3′-kinase, MAP kinase, and Syp have been implicated in mediation of diverse responses to many different growth factors and cytokines (117-119), it is therefore probable that an insulin-specific step may exist and defects in this step may be related with insulin resistance at post-receptor events.

D. Intracellular calcium in insulin action and insulin resistance

The roles of intracellular calcium ($[Ca^{2+}]i$) in insulin action has been previously proposed by several studies (reviewed in ref. 118), although not all studies support this concept. For instance, Klip et al. (121) and Kelly et al. (122) failed to show involvement of $[Ca^{2+}]i$ in insulin action on muscle cells and adipocytes.

Ca^{2+} involvement in insulin action may be expressed at various levels of cellular metabolism. The insulin receptor contains a high-affinity Ca^{2+}-binding site and its phosphorylation is Ca^{2+}-dependent (123). It also has a calmodulin domain (124). In the

adipocyte plasma membrane, insulin increases both Ca^{2+} binding (125) and the high-affinity binding sites for calmodulin, and via its receptor kinase, it also stimulates calmodulin phosphorylation (126-128). In turn, calmodulin enhances insulin-evoked phosphorylation of the insulin receptor β-subunit (127).

Glucose transport in adipocytes is Ca^{2+}-dependent (129, 130) and so is liver glucose-6-phosphatase activity (131). Insulin has been found capable of increasing $[Ca^{2+}]i$ in adipocytes (132, 133), possibly indicating Ca^{2+} as a signal for insulin-mediated processes. Draznin et al. (133) have proposed that insulin-mediated glucose transport is maximized only within an optimal range of $[Ca^{2+}]i$, and those elevations of basal $[Ca^{2+}]i$ beyond this range can cause insulin resistance. Furthermore, they have suggested that insulin resistance in obese and NIDDM patients results from high basal $[Ca^{2+}]i$ in adipocytes, and showed that it can be reversed *in vitro* by treatment with the Ca^{2+}-channel blocker verapamil (133). In support of this concept, Byyny et al. (134) found that calcium channel blockade with nitrendipine in obese-elderly-hypertensive-patients resulted in significant increases in insulin-stimulated 2-deoxyglucose uptake in adipocytes obtained from abdominal wall biopsies. In contrast, Kelly et al. (122) could not demonstrate a relation between a rise in $[Ca^{2+}]i$ in adipocytes and insulin resistance; however, they did observe a strong correlation between Ca^{2+} influx and inhibition of insulin-dependent glucose transport. Therefore, although further research is needed to establish the mechanisms by which $[Ca^{2+}]i$ produces insulin resistance, the evidence indicates that abnormal cellular calcium handling, particularly sustained elevation in $[Ca^{2+}]i$ may be involved in insulin resistance. This, in turn, may be due to a diminished ability of insulin to

appropriately modulate $[Ca^{2+}]i$ in cells with pre-existing elevations. Although the mechanisms that lead to such increases are not yet well understood, they appear to include an enhanced influx of calcium via calcium channels.

E. Effects of calcium channel blockade on insulin resistance and obesity

A large body of evidences has demonstrated an improvement of insulin sensitivity by calcium channel blockers. Voltage-dependent, L-type, calcium channel complexes contain at least three distinct binding sites: the dihydropyridine site, at which drugs such as nitrendipine, nifedipine, nimodipine and isradipine bind, the penylalkylamine site at which drugs such as verapamil and flunarizine act, and the benzothazepine site at which diltiazem binds (135, 136). The phenylalkylamine and benzothazepine binding sites are allosterically linked to the dihydropyridine site. Diltiazem increases the binding of dihydropyridines at their receptor sites and dihydropyridine calcium channel antagonists allosterically increase diltiazem binding, while verapamil showed complex biphasic inhibition of dihydropyridine binding (136-139).

1. Animal studies

Treatment with nitrendipine for 3 weeks decreased fasting glucose level and significantly improved glucose tolerance in non-obese spontaneously hypertensive rats without a change of body weight (140). This improved glucose tolerance appeared to be due to an increase in glucose uptake in muscle (140).

Benidipine, a dihydropyridine calcium antagonist, decreased body weight and body fat in mice made obese by pretreatment with monosodium-L-glutamate (MSG) (141). Benidipine appears to activate brown adipose tissue to induce body weight loss (141).

Nifedipine treatment for one month significantly decreased body weight compared to controls and three month treatment resulted in decrease in total, abdominal, and subcutaneous fat masses in nine-month-old obese female and male SHHF/Mcc-*fa*^{cp} rats (142). Nifedipine also reduced plasma triglyceride and fasting glucose levels and improved in insulin response to an oral glucose load in obese females but was without effects in obese males of SHHF/Mcc-*fa*^{cp} rats (142).

2. Clinical studies

Two different types of calcium channel antagonist, nitrendipine and diltiazem improved glucose tolerance, lowered fasting and glucose-stimulated circulating insulin levels in insulin-resistant obese and hypertensive patients (143, 144). Obese hypertensive elderly patients demonstrated marked hyperinsulinemia and reduced submaximally stimulated 2-deoxyglucose (DG) uptake in adipocytes which exhibited an elevated [Ca^{2+}]i (134). One month of therapy with nitrendipine reduced plasma insulin to control values during an oral glucose tolerance test in obese hypertensive individuals and restored 2-DG uptake at submaximally effective insulin concentrations to control values in normal weight and obese hypertensive subjects (134). Similarly, the long-acting Ca^{2+}-channel blocker amlodipine reduced steady-state plasma glucose and improved insulin sensitivity in essential hypertensive subjects (145). Further, verapamil normalized metabolic derangements, including glucose uptake, glycogen synthase and glycogen content, in hemodialysis patients who were characterized by increased basal [Ca^{2+}]i in polymorphonuclear leukocytes (146).

F. Mechanisms by which increased intracellular calcium produces insulin resistance

1. Attenuation of dephosphorylation and activation of insulin-sensitive substrates including glucose transporter-4 and glycogen synthase

The molecular mechanism(s) by which increased $[Ca^{2+}]i$ induces insulin resistance is poorly understood, but a number of potential candidate steps at which metabolic control of glucose disposal may occur can be identified. These include any of the rate-limiting steps in glucose metabolism, such as (1) insulin receptor binding (and subsequent kinase activity), (2) glucose transport across the cell membrane, (3) glucose oxidation, and (4) glycogen synthesis. The effects of $[Ca^{2+}]i$ on insulin binding and insulin receptor kinase activity have been addressed in studies using adipocytes with normal and elevated $[Ca^{2+}]i$ (147). The results of these studies do not distinguish any unambiguous change in insulin binding or tyrosine kinase activity of the insulin receptor in cells with high levels of cytosolic calcium (147).

Several investigators have demonstrated impaired stimulation of the glucose transport system in adipocytes and muscle strips of obese individuals and NIDDM patients (148-150). The transport of glucose across animal cell membranes is catalyzed by members of two distinct gene families (151). The facilitated-diffusion glucose transporters are ubiquitously expressed in mammalian cells (152), whereas the Na^+/glucose cotransporters appear to be restricted to selected epithelial cells of renal tubules and the intestinal mucosa (153). The Na^+-dependent proteins are secondary active-transport systems that reside in the apical membranes of the epithelia and concentrate glucose from the intestinal contents and the forming urine. The facilitated-diffusion transporters are

passive systems that equilibrate sugar across membranes. The latter proteins are responsible for the movement of sugar from the blood into cells, supplying cellular glucose for energy metabolism and the biosynthesis of sugar-containing macromolecules.

Glucose transport by facilitated diffusion is mediated by a family of tissue-specific membrane glycoproteins. To date, six genes encoding homologous but distinct glucose transporter (GLUT) proteins, and one pseudogene that does not encode protein, have been identified and designated GLUT1-7, based on the order in which they were cloned (154, 155). These glucose transporter proteins are highly homologous integral membrane proteins with 12 membrane-spanning domains, a single glycosylation site, and cytoplasmic NH_2 and COOH termini.

GLUT 4 is the insulin-responsive transporter isoform and is expressed exclusively in tissues that exhibit acute activation of glucose uptake by insulin, mainly adipose and skeletal muscle tissues (156). GLUT 4 protein levels were reported to be depressed by 23 % in the obese nondiabetic and 18 % in the obese NIDDM individuals (157). However, Pederson et al. (158) and Handberg et al. (159) found that GLUT 4 levels are normal in vastus lateralis biopsied from NIDDM patients. Nonetheless, insulin stimulates a translocation of GLUT 4 from an intracellular membrane pool to the plasma membrane and increases the intrinsic activity of the GLUT 4 (160). The intrinsic activity of GLUT 4 depends on the phosphorylation state (161). Phosphorylation of serine residues (161) on GLUT 4 significantly diminishes its intrinsic activity (162). Insulin promotes dephosphorylation of GLUT 4 (163) through phosphoserine phosphatase 1 (PP1) which is

activated by an insulin-stimulated protein kinase (161). Further, okadaic acid, an inhibitor of phosphatase 1 and 2A, inhibits the insulin-stimulated glucose transport (164).

The phosphorylation status of GLUT 4 was significantly increased in adipocytes when cells were depolarized with K^+ or exposured to parathyroid hormone (PTH), both of which cause an increase in Ca^{2+} influx, while nifedipine reduced GLUT 4 phosphorylation toward control levels in K^+-treated cells (165). Similarly, a combination of a cAMP antagonist (RpcAMP) and nitrendipine restored GLUT 4 phosphorylation in PTH treated cells (165).

PP1 is a ubiquitous enzyme that exists in association with glycogen particles, myosin, or other subcellular components (166). So far, glycogen-associated PP1 has been studied most extensively and has been found to contain a regulatory, or glycogen-bound, subunit (G-subunit) and a catalytic subunit (C-subunit) (167). The regulatory G-subunit contains two serine phosphorylation sites ('site-1' and 'site-2'). When dephosphorylated the G-subunit binds the C-subunit, producing a less active protein. An insulin-stimulated protein kinase (ISPK-1) promotes phosphorylation of 'site-1' of the G-subunit, turning PP1 into a 'more active' state. In contrast, cAMP-dependent kinase (PKA) mediates full phosphorylation of both 'site-1' and 'site-2' of the G-subunit. PKA also phosphorylates and activates the specific inhibitor of PP1 (Inhibitor-1) and allows the inhibitor to bind to the C-subunit of PP1 (168-170). Accordingly the C-subunit of PP1 dissociates from the G-subunit and becomes inhibited. Three structurally similar, but genetically distinct isoforms of PP1 catalytic subunit (PP1α, PP1β, and PP1γ) were identified in different

mammalian tissues including skeletal muscle (171-174) and PP1β appeared to be the predominant glycogen-bound from in rabbit skeletal muscle (175).

Reduced basal and insulin-stimulated activity of PP1 was shown in skeletal muscle of insulin-resistant Pima Indians (176). Sustained elevations of $[Ca^{2+}]i$ induced by K^+ or PTH were also accompanied by inhibition of PP1 activity in insulin target cells (170, 177). However, which classes of PP1 are inhibited by high $[Ca^{2+}]i$ remains unknown.

This inactivation of PP1 mediated by high $[Ca^{2+}]i$ is, in part, due to phosphorylation and activation of inhibitor-1 (170). Phosphorylation of inhibitor-1 significantly increases in cells with elevated $[Ca^{2+}]i$, while calcium channel blockade with nitrendipine completely prevents increases in inhibitor-1 phosphorylation (170).

Impaired insulin-stimulated nonoxidative glucose metabolism is a frequent finding in patients with overt NIDDM (178), predominantly due to an impairment in glycogen synthesis rate in skeletal muscle (179). The first event in the biogenesis of glycogen in skeletal muscle is catalyzed by a protein tyrosine glycosyltransferase and involves the covalent attachment of glucose to a single tyrosine residue at position 194 on the priming protein, termed glycogenin (180-186). The nature of the tyrosine glucosyltransferase is, however, not known and it is also unknown whether this first step in glycogen formation is under hormonal control. Once the first glucose is attached to the glycogenin backbone the glucan chain from the tyrosine 194 residue is extended by sequential addition of up to seven further glucose residues. This process is mediated by glycogenin itself (Mn^{2+}/Mg^{2+}-dependent autoglycosylation). Glycogen synthase (GS) is then able to elongate the glucan

chain, but only when glycogenin and GS are complexed together. Moreover, it appears that GS dissociates from glycogenin during the later stages of glycogen biogenesis.

Glycogen formation and breakdown are controlled by GS and glycogen phosphorylase, respectively. Both enzymes are regulated covalently by phosphorylation/dephosphorylation. GS catalyzes the rate-determining reaction of glycogen synthesis, i.e., the transfer of glycosyl units from UDP-glucose to the glycogen molecule:

$$\text{UDP-glucose} + \text{glycogen}_n \xrightarrow[\text{GS}]{} \text{UDP} + \text{glycogen}_{(n+1)}$$

GS can exist as a dephosphorylated form of high activity or as less active phosphorylated form which is stimulated by glucose-6-phosphate (G-6-P), a well-known allosteric activator (187). In diabetic subjects, a defect in dephosphorylation (insulin resistance) may be compensated for by an increase in intracellular G-6-P. Dephosphorylation of GS is regulated by the serine phosphatase, PP1 (188).

Depolarizing rat adipocytes increased $[Ca^{2+}]i$ and subsequently reduced PP1 activity and the dephosphorylation of GS accompanied (189). Calcium channel blockade with nitrendipine or incubation with Ca^{2+} free medium completely restored dephosphorylation of GS, suggesting dephosphorylation of GS is regulated by $[Ca^{2+}]i$ (189).

Accordingly, high $[Ca^{2+}]i$ may induce insulin resistance by inhibiting the dephosphorylation of GLUT4, glycogen synthase and other insulin-sensitive substrates.

2. Negative feedback induced by protein kinase C

A substantial body of observations indicates that protein kinase C (PKC) plays a role in the insulin-evoked cellular response (190-192). Insulin increases diacylglycerol (DAG) and stimulates PKC activity in myocytes and adipocytes through the translocation of the enzyme to the plasma membrane and by an increase in its reaction velocity (190). Phobol esters, which stimulate PKC by binding to the DAG site of PKC, mimic insulin's effect on stimulation of 3-O-methylglucose (3-OMG) uptake in rat fat cells by stimulating phosphorylation of the β-subunit of the insulin receptor (193). Inhibitors of PKC blunt insulin-induced 2-DG uptake in rat adipocytes, while agonist of PKC directly stimulates glucose transport in BC3H-1 myocytes (191).

PKC also appears to play a role in regulatory feedback of insulin action by inhibiting the binding of insulin to its binding sites (194), the phosphorylation of the insulin receptors (195, 196), and the insulin-evoked stimulation of phospholipase C (197).

Insulin resistance could arise when PKC is stimulated by insulin-independent pathways, which in turn would blunt the response to insulin (198). Ca^{2+} is a key factor in the PKC translocation from the cytosol to the plasma membrane, "priming" PKC for interaction with DAG to assume the PKC active mode (199). Therefore, a rise in DAG and $[Ca^{2+}]i$ levels, singly or in combination, could enhance PKC activity through its translocation to the plasma membrane (200). Glucose can also stimulate PKC through a *de novo* synthesis of DAG, thereby promoting insulin resistance, and it can be reversed by PKC inhibition (201, 202).

To date, at least ten PKC isozymes have been identified but the physiologically important protein substrates have not yet been clearly identified (203). Isozymes of a, b and g (cPKCs) are Ca^{2+}-dependent and activated by Ca^{2+} (204). Therefore cPKC may be related to high $[Ca^{2+}]$i-mediated insulin resistance which is associated with insulin receptor β-subunit tyrosine kinase inactivation via phosphorylation.

VI. THE ANIMAL FATTY ACID SYNTHASE

Fatty acid synthase (FAS) is a rate limiting enzyme in the long term regulation of *de novo* lipogenesis (205), and its hyperactivity and overexpression is a key feature of obese rats (206, 207). Even in advance of the onset of hyperphagia and hyperinsulinemia, FAS is already increased by 50 % in preobese pup adipose tissue in Zucker obese rats (208), suggesting FAS as an appropriate obesity marker.

A. One gene, one polypeptide, seven enzymes

FAS is a multifunctional enzyme that catalyzes all the reactions in the conversion of acetyl CoA and malonyl CoA to long chain fatty acids. The synthesis of fatty acids *de novo* is achieved by the sequential condensation of two-carbon units derived initially from acetyl-CoA. The overall reaction sequence can be summarized by:

$$\text{Acetyl-CoA} + 7\ \text{Malonyl-CoA} + 14\ \text{NADPH} + 14\ H^+ \rightarrow$$

$$\text{Palmitic Acid} + 7\ CO_2 + 8\ \text{CoA} + 14\ NADP^+ + 6\ H_2O$$

The pathway can be described as a cyclic process in which an acetyl primer undergoes a series of decarboxylative condensations with seven malonyl moieties; each condensation reaction generates a 3-ketoacyl moiety that undergoes the same three-step β-carbon reduction to give a fully saturated acyl moiety two carbon atoms longer than in the previous cycle. Thus, after completion of seven cycles of elongation and reduction, the final product is the saturated C_{16} fatty acid, palmitic acid (209).

FAS comprises two multifunctional polypeptide chains with a subunit M_r 250,000, each containing seven discrete functional domains, including ketoacyl synthase, malonyl/acetyl transferase, dehydrase, enoylreductase, ketoreductase, acyl carrier protein

(ACP), and thioesterase; these are juxtaposed head-to-tail such that two separate centers for fatty acid assembly are formed at the subunit interface (209).

For initiation of fatty acid synthesis, an acetyl and a malonyl moiety are translocated from their CoA thioesters to specific thiol sites on the FAS through malonyl/acetyl transferase domain of FAS: the acetyl moiety to the ketoacyl synthase and the malonyl moiety to the ACP domain. Condensation of acetyl and malonyl moieties is accompanied by the loss of C-3 (as CO_2) and then the resulting four-carbon acyl moiety undergoes reduction, dehydration, and final reduction- catalyzed by three discrete enzyme components of the FAS, the ketoreductase, dehydrase, and enoyl reductase, forming a saturated acyl moiety. In preparation for the next cycle of elongation, the saturated acyl moiety is then transferred back to the ketoacyl synthase and the cycle of condensation, ketoreduction, dehydration, and enol reduction is repeated with another malonyl moiety. The final palmitoyl moiety is then released as a free fatty acid through the action of the resident thioesterase of FAS.

The cDNA sequences encoding the rat (210-213), goose (214), chicken (215, 216), mouse (217), and human (218) FASs have been reported, and the human FAS gene has been mapped to chromosome 17q25 (218).

B. Regulation of fatty acid synthase concentration by nutrients and hormone

FAS concentration has been known very sensitive to nutritional and hormonal status, although the enzyme activity is not regulated by any allosteric effectors or covalent modification (205, 219).

Dietary regulation of FAS concentration is mediated primarily by controlling transcriptional activity of the gene. Starvation causes comparable decreases in the synthesis rate, mRNA abundance, and transcriptional rate of hepatic FAS in rodents and geese; feeding a high-carbohydrate diet reverses these effects (220-229). Dietary polyunsaturated fatty acids are potent inhibitors for transcription of hepatic FAS in rats (230-232).

It has been known that an increase in plasma insulin level is directly related to increased FAS activity in the liver of rat pups reared on formula rich in carbohydrate (233). Streptozotocin-induced diabetic rats exhibit a decreased hepatic FAS synthesis rate, which restored with insulin (234). Glucagon or dibutyryl cAMP decreases in the synthesis of FAS; the effects of insulin and glucagon on the synthesis of FAS *in vivo* are associated with similar changes in the abundance of FAS mRNA (226, 227). Regulation of the level of FAS mRNA by these hormones is largely mediated by alterations in gene transcription, and the effect of insulin on the FAS mRNA level is abolished by cycloheximide administration, suggesting that ongoing protein synthesis is required for the transcriptional activation of the FAS gene by insulin (228). The cis-acting insulin-responsive element has been found within 70 bp of the transcription start site (235).

Thyroid hormone (3,5,3′-Triiodothyronine, T_3) affects FAS mRNA levels in a tissue specific manner; FAS mRNA levels are higher in hyper- than hypothyroid liver of rats and in white adipose tissue mRNA expression is increased by hyperthyroidism, while in brown adipose tissue, highest levels are recorded in hypothyroid animals (236).

Nutritional and hormonal regulations of FAS are also observed in cultured or primary adipocytes or hepatocytes. In fully differentiated 3T3-L1 adipocytes, the mRNA expression of FAS is stimulated by insulin and T_3 and inhibited by dibutyryl cAMP (227, 237). The stimulatory effect of T_3 on FAS mRNA abundance is due to an increase in transcription of the gene (237). In primary cultures of hepatocytes from adult mouse or rat, insulin and T_3 enhance and glucagon inhibits the synthesis rate of FAS (238, 239). T_3 causes comparable increases in mRNA abundance and transcription of the FAS gene, indicating that primary regulation is transcriptional and requires on-going protein synthesis and protein phosphorylation, as inhibitors of protein synthesis or phosphorylation block the T_3 stimulation effect on FAS transcription (240-242).

In primary cultured rat adipocytes, glucose causes a five- to seven-fold increase in FAS mRNA (243). In contrast, 3-O-methylglucose, a glucose analogue that is transported into the cell but not metabolized, was without effect on FAS mRNA levels; however, 2-deoxyglucose, a glucose analogue that is phosphorylated to 2-deoxyglucose-6-phosphate, stimulates the expression of FAS mRNA. This suggests that glucose-6-phosphate or a product derived therefrom is involved in mediating the glucose-induced stimulation of FAS mRNA in adipose tissue (243). The glucose-induced increase in FAS mRNA in Hep G2 cells is due to changes in the stability of mRNA (244).

Fatty acids, including octanoate (C8:0) and hexanoate (C6:0), inhibit the T_3-induced stimulation of transcription of the FAS gene in chick embryo hepatocytes in culture (245). Arachidonate (C20:4) inhibits the stimulation of FAS mRNA level caused by a combination of insulin, dexamethasone, and T_3 in cultured rat hepatocytes (246).

Transcription appears to be the regulated step, as dietary arachidonate inhibits transcription of the FAS gene in livers of intact rats (230).

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PART 3

EXPERIMENTAL INVESTIGATIONS

I. AGOUTI REGULATION OF INTRACELLULAR CALCIUM: ROLE IN THE INSULIN RESISTANCE OF VIABLE YELLOW MICE[1]

A. Abstract

Several dominant mutations at the agouti locus in the mouse cause a syndrome of marked obesity, hyperinsulinemia and insulin resistance. Although it is known that the agouti gene is expressed in an ectopic manner in these mutants, the precise mechanism by which the agouti gene product mediates these effects is unclear. Since intracellular calcium ($[Ca^{2+}]i$) is believed to play a role in mediating insulin action and dysregulation of calcium flux is observed in diabetic animals and humans, we examined the status of $[Ca^{2+}]i$ in mice carrying the dominant agouti allele, viable yellow (A^{vy}). We show here that in mice carrying this mutation, $[Ca^{2+}]i$ levels are elevated in skeletal muscle and the degree of elevation is closely correlated with the degree to which the mutant traits are expressed in individual animals. Moreover, we demonstrate that the agouti gene product is capable of inducing elevated $[Ca^{2+}]i$ levels in cultured and freshly isolated skeletal myocytes from wild-type mice. Based on these findings, we present a model in which we propose that the agouti polypeptide promotes insulin resistance in mutant animals through its ability to increase $[Ca^{2+}]i$.

[1]This manuscript has been published in similar form with co-authors Zemel, M. B., Woychik, R. P., Michaud, E. J., Kadwell, S. H., Patel, I. R., and Wilkison, W. O. in: Proc. Natl. Acad. Sci. USA 1995; 92: 4733-4737.

B. Introduction

The mouse agouti gene is normally involved in regulating the production of pigment granules that give rise to the wild-type coat color, which consists of black hairs with a subapical band of yellow (1, 2). Several dominant mutations at agouti, most notably lethal yellow (A^{y}) and viable yellow (A^{vy}), cause mice to develop a predominantly yellow coat color and to become obese, insulin resistant and hyperinsulinemic with age (reviewed in 3, 4). These mutants are collectively often called "yellow obese" mutants.

The agouti gene has been cloned and shown to encode a 131 amino acid protein with a consensus signal peptide (5, 6). Agouti is normally expressed in the skin during hair growth (5). Bultman et al. (5) discussed a model for the function of the agouti protein that was recently validated at the molecular level (7). Agouti functions in a paracrine manner to regulate the differential production of melanin pigments by the melanocyte (1, 2). Normally, α-melanocyte stimulating hormone (α-MSH) binds to its receptor on the melanocyte, activates adenylate cyclase, and thereby causes an increase in intracellular cAMP levels which stimulates production of eumelanin (black pigment) (8). However, when agouti is present within the hair follicle, it appears to block the ability of α-MSH to activate its receptor, thereby inhibiting cAMP production and causing a shift from eumelanin to phaeomelanin (yellow pigment) production (7).

While it is becoming increasingly clear how the agouti protein functions in the hair follicle, much less is known about how the agouti gene causes obesity and insulin resistance in the yellow mutants. Molecular analysis of A^{y} (5, 9, 10), A^{vy} (11), and a new dominant allele of agouti called A^{iapy} (12), revealed that in all cases, the agouti gene,

which is normally expressed in the developing hair follicle, has been modified in a manner that causes it to be expressed in most, if not all, tissues of the animal. Although agouti is being expressed with an altered tissue distribution, it appears to have retained the ability to produce a normal agouti protein. Agouti is a secreted molecule; however, agouti appears to function in a localized manner (2). Accordingly, the site of synthesis of the agouti protein is an important factor to consider in evaluating its biological activity in the animal. In this regard, it is presently unclear if it is the ubiquitous expression of agouti *per se*, or perhaps the ectopic expression of agouti in a specific tissue that is directly responsible for the development of obesity and the other dominant pleiotropic effects.

Because the yellow obese mutants are hyperinsulinemic and insulin resistant, and because type I muscle fibers (e.g., soleus muscle) are a primary target for insulin action, we considered the possibility that the muscle is responsive to the action of the agouti protein in yellow obese mutants. There are multiple potential cellular sites of insulin resistance; one such site is dysfunctional regulation of $[Ca^{2+}]i$. Elevations in $[Ca^{2+}]i$ have been shown to result in insulin resistance in several systems (13-19), although the relationship between $[Ca^{2+}]i$ and insulin signal transduction is complex and poorly understood. Accordingly, we initiated a series of experiments to measure $[Ca^{2+}]i$ levels and transport in insulin-sensitive tissue (skeletal muscle) of mice carrying the A^{vy} allele of agouti, and to determine the role of the agouti protein in regulating $[Ca^{2+}]i$. We report herein that adult A^{vy}/a mice (where *a* refers to non-agouti) exhibit increases in soleus Ca^{2+} influx and $[Ca^{2+}]i$ that correlate well with the degree of obesity in the animals. We further demonstrate that conditioned medium containing recombinant agouti protein stimulates

significant increases in $[Ca^{2+}]i$ over the baseline in both freshly isolated and cultured skeletal myocytes.

C. Materials and Methods

Animals: C57BL/6J-A^{vy} mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained at the Oak Ridge National Laboratory (Oak Ridge, TN) by mating A^{vy}/a mice to a/a siblings. Experiments were conducted on three to five-month old male and female viable yellow (A^{vy}/a) mice exhibiting either pseudoagouti, mottled, or yellow coat colors (see results) and were compared with age-matched non-agouti black (a/a) mice.

Preparation of isolated skeletal myocytes: Isolated soleus and gastrocnemius myocytes were prepared essentially as described by Beam et al. (20). Briefly, tissue was isolated from animals following an overnight fast and gently teased apart along the longitudinal axis. The tissue was then incubated at 37 °C for 40 min in a HEPES-buffered salt solution (HBSS; composition in mM: NaCl, 138; $CaCl_2$, 1.8; $MgSO_4$, 0.8; NaH_2PO_4, 0.9; $NaHCO_3$, 4; glucose, 25; glutamine, 6; HEPES, 20; and 0.5 % bovine serum albumin (BSA)) containing collagenase (type I, 2 mg/mL). After filtration and centrifugation, the cell pellets were resuspended in HBSS for measurement of $[Ca^{2+}]i$.

Preparation of cultured L6 myocytes: L6 skeletal myocytes were purchased from American Type Culture Collection (ATCC, Rockville, MD) in passage 4. Cells (1.8×10^6) were plated in a 150 cm^2 flask containing Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5 % calf serum, 5 % fetal bovine serum, 50 U/mL penicillin, 5 μg/mL streptomycin and 10 % glucose and maintained in a 5 % CO_2 ,100 % humidity

atmosphere. For sequential passage, non-confluent cells were rinsed with a Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution (Sigma, St. Louis, MO) and treated with 0.5 mg/mL trypsin for 2 min. Released cells were recovered by centrifugation. For $[Ca^{2+}]i$ determination, cells were trypsinized and resuspended in HBSS at a density of approximately 10^6 cells/mL.

Expression of murine agouti cDNA: A 707 bp EcoRV/PstI fragment of the full length agouti cDNA (6) was subcloned into a SmaI/PstI site in the baculoviral expression vector pVL1393 (Pharmingen, San Diego, CA) and the construct was verified by sequencing. This construct was then packaged and titered using standard methods (21). *Trichiplusia ni* (*T. ni*) cells were then infected at a multiplicity of infection of 2, and the medium was collected 48 hr after infection. This medium was then filtered with a 5 kDa cutoff Sartorius filter and used directly ("agouti-conditioned medium"). Controls consisted of medium alone and medium collected 48 hr after infection from *T. ni* cells infected with the wild-type baculovirus ("control medium"). Rabbit anti-peptide antibodies were generated against a fragment of murine agouti comprising the predicted amino acid residues from position 25 to position 40 (6). Samples of control and conditioned medium were electrophoresed on a 4-20 % SDS/PAGE gel and blotted to nitrocellulose. Western blots were performed with the agouti antipeptide antibody in 50 mM Tris, pH 7.5/150 mM NaCl/3 % BSA (fraction 5, Sigma, St. Louis, MO) at a 1:500 dilution. The second antibody was goat anti-rabbit IgG conjugated to alkaline phosphatase at 1:3000.

Northern blot analysis: Total cellular RNA from all tissues was extracted by using the guanidine thiocyanate procedure (22), enriched for poly(A)$^+$ RNA by using an oligo(dT)-cellulose column (23), electrophoresed through formaldehyde gels, and blotted to GeneScreen (DuPont) by standard procedures (22). The radiolabeled agouti probe (6) was prepared with the random hexamer labeling technique (24). Posthybridization filter washes were conducted at high stringency (in 0.03 M NaCl/0.003 M sodium citrate/0.1 % SDS at 68 °C).

[Ca^{2+}]i determination: [Ca^{2+}]i levels in freshly isolated soleus and gastrocnemius myocytes and in suspensions of L6 myocytes were determined spectrofluorometrically in fura-2 loaded cells as previously described (25, 26). Briefly, cell suspensions were loaded with fura-2-acetoxymethyl ester in the dark for 20 min at 37 °C with shaking, washed with HBSS, and resuspended immediately prior to [Ca^{2+}]i determination. [Ca^{2+}]i was measured by using dual excitation (340 and 380 nm) and single emission (510 nm) fluorometry. Digitonin (25 μM) and Tris/EGTA (both 100 mM, pH 8.7) were used to determine maximal and minimal fluorescent ratios, respectively, and [Ca^{2+}]i was then calculated from fluorescent ratios using the equation of Grynkiewicz et al. (27). To evaluate the effects of the agouti-conditioned medium, cells were preincubated in a 1:9 (v/v) mixture of conditioned or control medium and HBSS for 40 min, washed, resuspended and loaded with fura-2 as above.

$^{45}Ca^{2+}$ efflux and influx: Soleus $^{45}Ca^{2+}$ efflux and influx were determined by using slight modifications of methods previously described (28). For efflux, the soleus was

loaded with $^{45}Ca^{2+}$ by incubation in a physiological salt solution (PSS) containing 1 μCi (37 kBq) $^{45}Ca^{2+}$/mL while being gassed with 95 % CO_2/5 % O_2 at 37 °C. The washout of radioactivity into unlabelled PSS was then followed with 3 min intervals for 90 min. $^{45}Ca^{2+}$ efflux was expressed as a percentage of the original $^{45}Ca^{2+}$ load remaining in the tissue at each time point (28), and the Ca^{2+} efflux rate constant was then calculated from the $^{45}Ca^{2+}$ efflux curve. To determine Ca^{2+} influx, soleus segments were equilibrated in PSS for 10 min at 37 °C while being gassed with 95 % CO_2/5 % O_2. They were then transferred to PSS containing 1 μCi $^{45}Ca^{2+}$/mL for 2-10 min to measure rate of Ca^{2+} influx.

Statistical analysis: Comparisons between A^{vy}/a and a/a mice or between agouti-conditioned and control medium were evaluated via Student's *t* test. Comparisons among black, pseudoagouti, mottled and yellow animals were evaluated via one-way analysis of variance. The effects of agouti-conditioned versus control medium on $[Ca^{2+}]i$ in the presence or absence of extracellular Ca^{2+} were assessed by two-way (incubation medium x treatment) analysis of variance. The relationship between $[Ca^{2+}]i$ and body weight was determined via linear regression analysis.

D. Results

To evaluate the effect of the ectopic expression of the agouti gene on $[Ca^{2+}]i$, we chose to use mice carrying the A^{vy} mutant allele as our model animal. A^{vy}/a mice are especially well suited for these experiments because the agouti gene is ectopically expressed at various levels in individual animals, and the level of agouti expression correlates with the degree to which the animals express the mutant phenotype (Fig. 1). For example, $A^{vy}/-$ mice with high or moderate levels of ectopic agouti expression have

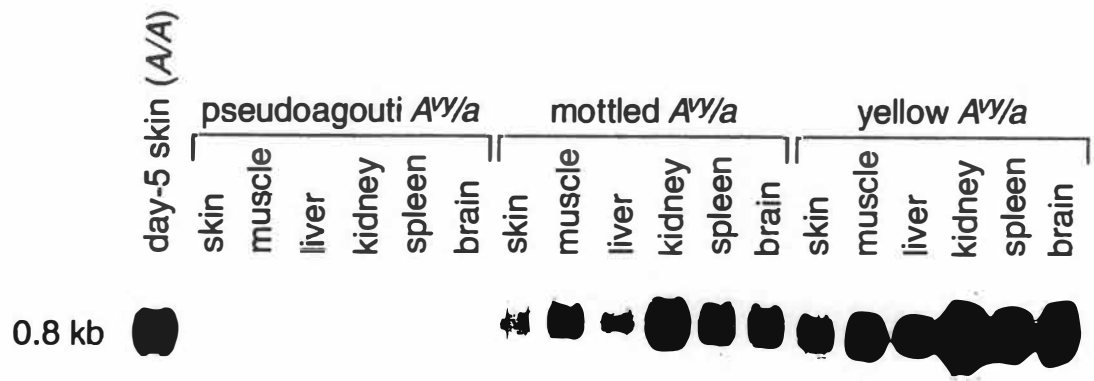

Figure 1. Northern blot analysis of agouti locus expression in various tissues from adult viable yellow (A^{vy}/a) mice exhibiting either a completely pseudoagouti, moderately mottled (pseudoagouti plus yellow mix), or solid yellow coat color. Neonate skin from a day 5 wild-type agouti (A/A) mouse was included as a positive control. A wild-type agouti cDNA clone was ^{32}P-labeled and hybridized to these poly $(A)^+$ RNAs (2.5 μg per lane, except for the following: mottled skin, 1.4 μg; mottled muscle, 2.0 μg; and yellow skin, 2.0 μg).

completely yellow fur or are mottled with patches of agouti-like hair mixed with totally yellow hair, respectively; these animals have a high propensity to develop the obesity and hyperinsulinemia traits. On the other hand, mice that ectopically express agouti at very low levels have a coat color that is similar to the wild-type agouti color and are referred to as pseudoagouti. Pseudoagouti mice have normal body weights and are not hyperinsulinemic, suggesting that there may be a threshold level at which agouti exerts these effects. Therefore, the effect of agouti on $[Ca^{2+}]i$ and insulin resistance can be studied in sibling $A^{vy}/-$ mice that differ only in their level of agouti expression, and their coat colors provide a general indication of the level of ectopic agouti expression and their propensity to become obese and hyperinsulinemic.

This study was conducted in a muscle consisting primarily of insulin-sensitive type I muscle fibers (soleus) as well as in a muscle containing primarily less insulin-sensitive type II fibers (white gastrocnemius). Collectively, A^{vy}/a of both genders exhibited 37 % greater soleus $[Ca^{2+}]i$ compared to non-agouti black controls ($p < 0.01$). However, this difference was dependent upon the degree of phenotypic expression of the A^{vy} genotype; soleus $[Ca^{2+}]i$ in the pseudoagouti animals was not significantly different from that in a/a mice, whereas a two-fold elevation was found in the yellow mice (Table 1). The mottled A^{vy}/a animals exhibited levels only slightly lower than the yellow mice. These variations in $[Ca^{2+}]i$ closely tracked the heterogeneity in body weight, and there was a high degree of correlation between the two ($r = 0.91$, $p < 0.01$; Fig. 2). These data are also consistent with the previous observations that pseudoagouti mice do not become obese, whereas both mottled and yellow mice have the propensity to become severely obese.

Table 1. $[Ca^{2+}]i$ and Ca^{2+} flux in non-agouti black (*a/a*) and viable yellow (A^{vy}/a) mice with various levels of ectopic agouti expression

Measurement	Nonagouti black mice	Viable yellow mice: Pseudo-agouti	Viable yellow mice: Mottled	Viable yellow mice: Yellow
$[Ca^{2+}]i$, nM				
Soleus	174 ± 6	177 ± 8	308 ± 35[a]	330 ± 39[a]
Gastrocnemius	293 ± 28	283 ± 29	476 ± 36[a]	350 ± 40
$^{45}Ca^{2+}$ flux (Soleus)				
Efflux rate[1]	11.9 ± 3.2	ND	ND	10.1 ± 1.8
Influx rate[1]	115 ± 28	ND	ND	166 ± 32[a]

Data are reported as mean ± SE.

ND, no data

[1]Unit for the efflux and influx rates are as follows: efflux, min^{-1}; influx, cpm/ng of protein/min

[a]$p < 0.01$ vs. non-agouti black mice

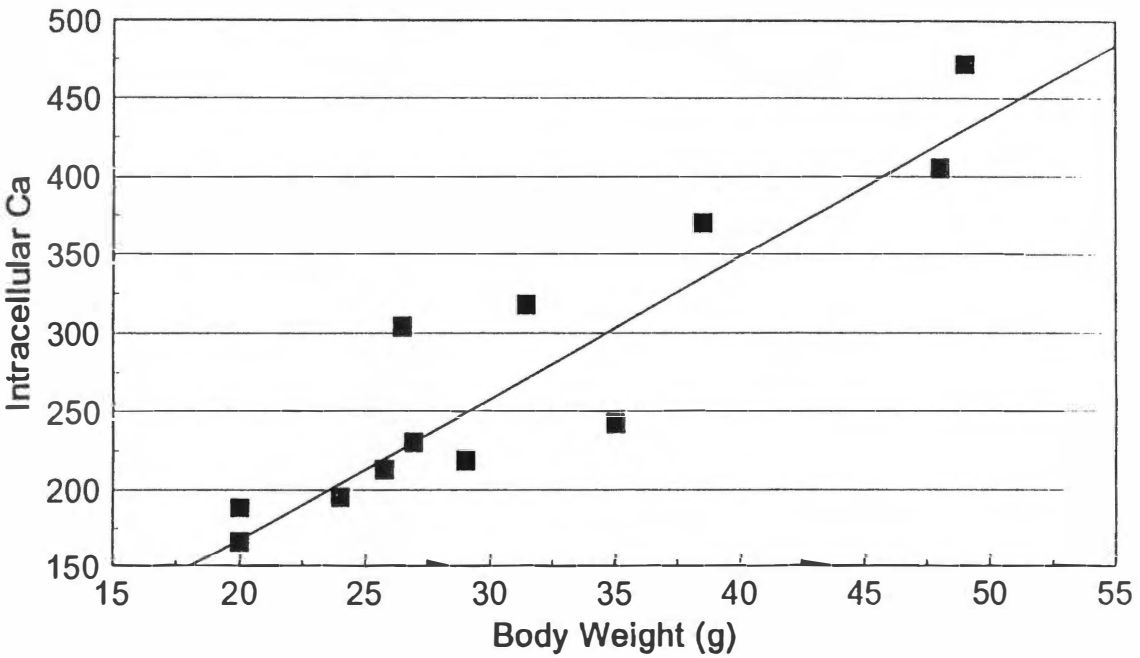

Figure 2. Relationship between body weight and $[Ca^{2+}]i$ in freshly isolated soleus myocytes from viable yellow ($A^{vy}/-$) mice with either a pseudoagouti, mottled or yellow coat color. There is a significant correlation ($r = 0.91$, $p < 0.01$; $n = 18$) between $[Ca^{2+}]i$ and body weight.

In gastrocnemius, $[Ca^{2+}]i$ was increased only in male mottled and yellow mice compared to the non-agouti control black mice (Table 1), and there was no significant relationship between body weight and $[Ca^{2+}]i$ in this muscle type (data not shown).

To evaluate the cause of the increased $[Ca^{2+}]i$, Ca^{2+} efflux and influx studies were conducted in soleus. Basal Ca^{2+} efflux rate was not significantly different between A^{vy}/a and a/a mice (Table 1), although insulin-stimulated efflux was diminished (data not shown), consistent with a diminution in Ca^{2+}-ATPase activity in insulin resistance (26, 28, 29). In contrast, the basal Ca^{2+} influx rate was significantly increased in A^{vy}/a mice (Table 1).

To directly evaluate the role of the agouti gene product in regulating skeletal muscle $[Ca^{2+}]i$, we prepared conditioned media containing recombinant agouti protein. For this purpose, the wild-type agouti cDNA was subcloned into a baculoviral expression vector and *T. ni* cells were infected with either the agouti expression baculovirus or a wild-type baculovirus control. The medium collected from *T. ni* cells infected with the agouti expression baculovirus produced a polypeptide that reacted against an agouti anti-peptide antibody (Fig. 3). The controls, including medium collected from mock-infected *T. ni* cells and medium collected from *T. ni* cells infected with a wild-type baculovirus, showed no such immunoreactive species. In the agouti-conditioned medium, the antibody was completely blocked by incubation with the peptide antigen (data not shown), and the preimmune serum did not react with the agouti-containing medium. Additionally, the medium had agouti biological activity, since it was used to antagonize the ability of α-MSH to stimulate cAMP production in B16f10 melanoma cells (7).

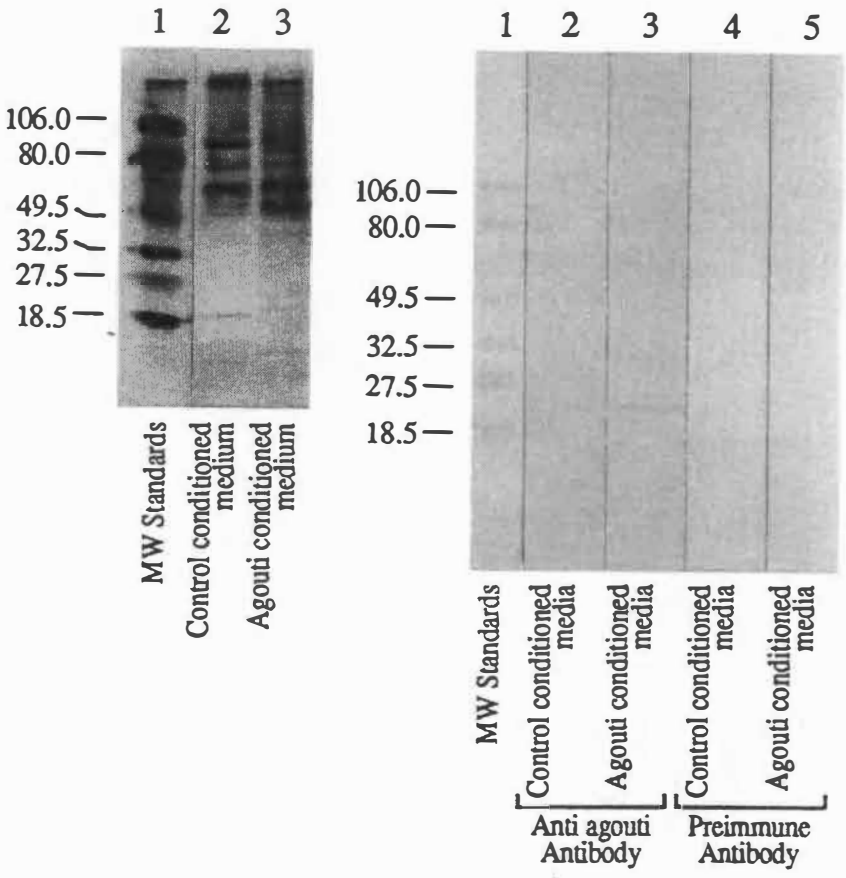

Figure 3. Expression of the murine agouti peptide. Ten micrograms of medium protein from *T. ni* cells 48 hr after infection of either a wild-type baculovirus or the agouti expression baculovirus was loaded onto a 4-20% SDS/PAGE gel (NOVEX, San Diego) and silver-stained. A duplicate gel was transferred to nitrocellulose and probed with either an agouti anti-peptide antibody or preimmune antibody. (Left) Silver-stained gel of the conditioned medium. The agouti polypeptide is not readily apparent. (Right) Western blot of a duplicate gel. The anti-agouti peptide antibody detects a protein of 21kDa.

Because the medium is highly fluorescent, acute effects of agouti-conditioned medium on $[Ca^{2+}]i$ were not studied. Instead, L6 myocytes or freshly isolated myocytes were incubated in agouti-conditioned or control medium for 40 min and washed, and thenbasal $[Ca^{2+}]i$ of the cells was measured. This 40-min incubation period prior to $[Ca^{2+}]i$ measurement was comparable to the time required to isolate and study mouse soleus and gastrocnemius myocytes. The agouti-conditioned medium caused a significant increase in $[Ca^{2+}]i$ in L6 cultured myocytes (Table 2) and in soleus myocytes isolated from non-agouti black mice (data not shown). However, myocytes isolated from A^{vy}/a mice, in which $[Ca^{2+}]i$ was already elevated, exhibited no further increase after incubation in agouti-conditioned medium (data not shown). Agouti-mediated increases in $[Ca^{2+}]i$ were dependent upon extracellular Ca^{2+}, as no such increase was found in Ca^{2+}-free HBSS (Table 2).

E. Discussion

Although the genetic defect in yellow obese mice involves the ectopic expression of the agouti gene, it was previously unclear which tissues were responding specifically to the agouti gene product in a manner that causes the obesity and hyperinsulinemia/insulin resistance. It was also unknown how the agouti protein was effecting a response at the cellular level. Here we have determined that adult A^{vy}/a mice exhibit significant increases in soleus Ca^{2+} influx and $[Ca^{2+}]i$, and that these increases correspond with ectopic agouti expression and obesity. Moreover, conditioned medium containing recombinant agouti protein stimulated significant increases in $[Ca^{2+}]i$ in both freshly isolated and cultured skeletal myocytes. These data provide compelling evidence to support a direct role of

Table 2. Effects of agouti-conditioned medium on $[Ca^{2+}]i$ in L6 cultured skeletal myocytes in the presence or absence of extracellular Ca^{2+}

	Medium	
Treatment[1]	Control-conditioned	Agouti-conditioned
Ca^{2+}-containing	84.8 ± 3.3	123.2 ± 4.8[a]
Ca^{2+}-free	88.7 ± 4.0	95.7 ± 2.7

[1] n = 8 per group

[a] $p < 0.01$

agouti in modulating soleus responses to insulin. Accordingly, the action of agouti on type I muscle fibers in the yellow obese mutants may contribute to their insulin resistance.

Although the A^{vy}/a mice exhibited a decrease in insulin-stimulated soleus Ca^{2+} efflux, there was no decrease in basal efflux. This is consistent with our previous observations in rat aortic smooth muscle, in which impaired Ca^{2+}-ATPase-mediated Ca^{2+} efflux was a result, rather than a cause, of insulin resistance (30-32). In contrast, the A^{vy}/a mice did exhibit an increase in Ca^{2+} influx. Therefore, increased $[Ca^{2+}]i$ in mice carrying the A^{vy} allele of agouti appears to result from an increase in Ca^{2+} influx rather than from either an impairment in Ca^{2+} efflux or an increase in Ca^{2+} release from sarco/endoplasmic reticulum stores. This suggestion is further supported by the observation that the increase in $[Ca^{2+}]i$ in L6 skeletal myocytes in response to incubation in agouti-conditioned medium was dependent upon extracellular Ca^{2+} and did not occur in a Ca^{2+}-free HBSS. However, it is also possible that agouti-mediated effects on receptor interactions may be dependent upon the presence of extracellular Ca^{2+}.

Several lines of evidence support a role for Ca^{2+} in modulating tissue insulin sensitivity. Draznin et al. (13) demonstrated an optimal range of $[Ca^{2+}]i$ for maximizing insulin-stimulated glucose transport, with elevations beyond this range causing marked decreases in adipocyte insulin sensitivity. Similarly, data from a number of studies indicate that increasing $[Ca^{2+}]i$ in isolated adipocytes results in significant inhibition of insulin-stimulated glucose transport (17-19) and oxidation (14). In addition, Ca^{2+} entry blockade in obese elderly humans resulted in significant increases in peripheral insulin sensitivity (15, 16). Finally, Resnick et al. (33) reported that obese patients exhibited a 41 %

increase in $[Ca^{2+}]i$ compared with their lean counterparts. Moreover, there was a significant positive correlation between erythrocyte $[Ca^{2+}]i$ and body mass index in lean and obese subjects, similar to the correlation between soleus muscle $[Ca^{2+}]i$ and body weight observed in the present study (Fig. 2).

While the relationship between $[Ca^{2+}]i$ and insulin signal transduction is not well understood, it appears that elevations in $[Ca^{2+}]i$ may, in part, result in insulin resistance by affecting the phosphorylation of glucose transporter type 4 (Glut 4) and other insulin sensitive substrates within the cell (17-19). Glut 4 is the primary insulin-responsive glucose transporter in the cell and its activity is regulated by serine phosphorylation (34). Normally, insulin activates phosphoserine phosphatase 1 (PP1), which dephosphorylates and, hence, activates Glut 4 (34). Recently Reusch et al. (17) reported that K^+ depolarization or parathyroid hormone treatments increased $[Ca^{2+}]i$ in isolated adipocytes, and that this appeared to result in an increased phosphorylation of Glut 4. This effect was likely due directly to changes in $[Ca^{2+}]i$ because treatment with nitrendipine, which blocks entry of Ca^{2+} into the cell, maintained normal Glut 4 levels of phosphorylation. These effects of increased $[Ca^{2+}]i$ on phosphorylation of Glut 4 appeared to be mediated by Ca^{2+}-induced phosphorylation and activation of inhibitor 1, which functions to inhibit PP1 activity (18). Overall, based on these findings, we predict that the hyperinsulinemia/insulin resistance in the yellow obese mutants causes increased $[Ca^{2+}]i$ in soleus muscle which induces increased activity of inhibitor 1. This effect leads to an increase in phosphorylation of PP1, which, in turn, results in an increased phosphorylation and inactivation of the insulin-responsive substrates within the cell, including Glut 4.

Experiments to directly evaluate the activity of Glut 4 and the other insulin responsive components in the cell will help to confirm this prediction.

While the ectopic expression of agouti causes changes in $[Ca^{2+}]i$, it remains unclear how specifically agouti is signaling the responses in soleus muscle described in this report. Within the hair follicle, agouti acts as an antagonist for the binding of α-MSH to its receptor on the melanocyte (7). In this manner, it prevents the α-MSH-induced increases in the level of cAMP within the cell (7), and hence elicits its biological action. Since the α-MSH receptor does not appear to be expressed in skeletal muscle (35), agouti cannot be acting in exactly the same manner in muscle as it is in the hair follicle. Another member of the melanocortin receptor family, melanocortin 5 receptor (MC5R), is expressed on skeletal muscle (36). Therefore, it is possible that agouti is functioning on the MC5R in soleus muscle in a manner similar to that which occurs with the α-MSH receptor in the hair follicle. Alternatively, agouti may act through an interaction with another as yet unidentified receptor, or possibly by upregulating voltage- and/or receptor-operated Ca^{2+} channels within the cell. Additional experiments utilizing purified recombinant agouti protein should help to address this issue.

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II. AGOUTI INDUCED ELEVATION OF INTRACELLULAR CALCIUM. APPARENT INTERACTION WITH MELANOCORTIN RECEPTORS[1]

A. Abstract

Several dominant mutations at the murine agouti locus cause a syndrome of marked obesity and insulin resistance. We recently reported that intracellular free calcium ($[Ca^{2+}]i$) is elevated in viable yellow mice. Since $[Ca^{2+}]i$ has a key role in the pathogenesis of insulin resistance, obesity and hypertension, the role of the purified agouti gene product in regulating $[Ca^{2+}]i$ was evaluated in a number of cell types. Purified murine agouti induced slow, sustained increases in $[Ca^{2+}]i$ in L6 skeletal myocytes, A7r5 vascular smooth muscle cells and 3T3-L1 adipocytes in a dose-dependent fashion. In L6 skeletal myocytes, agouti stimulated an increase in $[Ca^{2+}]i$ with an apparent EC_{50} of 62 nM. This response was substantially inhibited by Ca^{2+} entry blockade with nitrendipine. To determine if melanocortin receptors may play a role in agouti regulation of $[Ca^{2+}]i$, we examined the effect of melanocortin peptide and agouti in cells stably transfected with human melanocortin receptors. Human embryonic kidney 293 (HEK-293) cells transfected with either the human melanocortin 1 or melanocortin 3 receptor responded to human agouti with slow, sustained increases in $[Ca^{2+}]i$, while non-transfected HEK-293 cells did not respond to agouti. This direct effect of agouti on stimulating increases in $[Ca^{2+}]i$ suggests a potential mechanism for agouti-induced insulin resistance.

[1]This manuscript has been submitted for publication in similar form with co-authors Kiefer, L. L., Woychik, R. P., Wilkison, W. O., Truesdale, A., Ittoop, O., Willard, D., Nichols, J. and Zemel, M. B. in: Am. J. Physiol.

B. Introduction

Insulin resistance is characterized by abnormal intracellular Ca^{2+} ($[Ca^{2+}]i$) homeostasis in several types of cells including skeletal myocytes, vascular smooth muscle cells, and adipocytes (1-3). Increasing $[Ca^{2+}]i$ level in the cell inhibits insulin-stimulated glucose transport and Ca^{2+} channel antagonism improves cellular insulin sensitivity (1, 3). Increased $[Ca^{2+}]i$ may contribute to insulin resistance by inhibiting the dephosphorylation of insulin-sensitive substrates, including glycogen synthase (4) and the insulin-sensitive glucose transporter (Glut 4) (5); this may result from Ca^{2+}-induced phosphorylation and activation of inhibitor-1, thereby blocking the activation of phosphoserine phosphatase-1 (6). It is also possible that Ca^{2+}-mediated increases in activity of protein kinase C may contribute to insulin resistance via phosphorylation of the insulin receptor β-subunit tyrosine kinase (7).

Dominant mutations at the agouti locus in the mouse such as lethal yellow (A^y) and viable yellow (A^{vy}) are obese and exhibit hyperinsulinemia at 5-6 week of age, prior to becoming significantly heavier than control (8, 9). Molecular analyses reveal that these mice ectopically express wild-type agouti transcripts, and that this is due to a mutation in the promoter region of the agouti gene (10-12). Transgenic mice in which the wild-type agouti cDNA is placed under the transcriptional control of ubiquitous gene promoters not only express the agouti mRNA in multiple tissues but also develop obesity, hyperinsulinemia, hyperglycemia and yellow coat color, demonstrating that ectopic

expression of agouti *per se* is responsible for the diseases associated with dominant agouti alleles (13).

While the ectopic expression of agouti is responsible for the dominant pleiotropic effects (e.g. obesity and insulin resistance) characteristic of A^{vy} mice, the mechanism of agouti action and its target tissue remains unclear. However, the mechanism of agouti regulation of mouse coat color has been described (14) and may serve as a paradigm for investigating the mechanism of agouti's effects on insulin sensitivity and obesity. Melanocytes in the hair bulb produce two types of pigment; eumelanin (black) and phaeomelanin (yellow), and normally, synthesis of the pigment can be switched between the two types (15). Melanocytes begin to produce eumelanin when α-melanocyte stimulating hormone (α-MSH) binds to its receptor on the cell membrane, stimulating adenylate cyclase (16). The agouti gene product antagonizes the binding of α-MSH, causing melanocytes to produce phaeomelanin (14). Receptors for several members of the melanocortin family have been identified, although their functions remain unclear. Accordingly, it is possible that similar inhibition of melanocortin peptide binding to one or more of these receptors may be responsible for the dominant pleiotropic effects of agouti.

We have recently demonstrated that A^{vy} mice exhibited a marked elevation of skeletal muscle $[Ca^{2+}]i$ which was highly correlated with the degree of ectopic overexpression of agouti and obesity (17). Further, we reported that incubation of cultured skeletal myocytes with media containing recombinant murine agouti protein caused an increase in steady-state $[Ca^{2+}]i$, and that this may contribute to the insulin resistance characteristic of mutations at the mouse agouti locus (17).

Consequently, we conducted this study to determine (a) the effects of purified recombinant agouti protein on $[Ca^{2+}]i$ in several distinct cell types, and whether (b) melanocortin receptors play a role in agouti mediated Ca^{2+} signaling. We report herein that the recombinant murine agouti protein induced elevation of $[Ca^{2+}]i$ in L6 skeletal myocytes, A7r5 vascular smooth muscle cells and 3T3-L1 adipocytes, and that this effect is sensitive to Ca^{2+}-channel blockade. Further, agouti regulation of $[Ca^{2+}]i$ appears to be modulated by melanocortin receptors in human embryonic kidney 293 cells (HEK-293 cells), although the role of melanocortin receptors in other cell types is not clear. This suggests that agouti may regulate $[Ca^{2+}]i$ in some tissue types via interaction with melanocortin receptors.

C. Materials and Methods

Cell culture: The spontaneously fusing L6 skeletal myocytes (ATCC, Bethesda, MD) were grown in 75-cm^2 flasks in Dulbecco's modified Eagle's medium with 10 % glucose (DMEM) supplemented with 5 % fetal bovine serum (FBS) (v/v) , 5 % calf serum supplemented iron-enriched (CS) (v/v), and antibiotics (50 units of penicillin/mL and 5 μg of streptomycin/mL). Cultures were maintained in continuous passages (< 20) by trypsinization (0.05 % trypsin) of semiconfluent nonfused cells. A7r5 vascular smooth muscle cells (ATCC, Bethesda, MD) were grown in the same conditions as that for L6 cells. 3T3-L1 cells (ATCC, Bethesda, MD) were grown in 100 mm dishes in DMEM containing 10 % FBS and antibiotics. For differentiation of fibroblasts into adipocytes, confluent cells were incubated in DMEM/FBS containing 0.5 mM isobutylmethylxanthine (IBMX) (Sigma, St. Louis, MO) and 0.25 μM dexamethasone (DEX) (Sigma, St. Louis,

MO) for 60 hr and then IBMX and DEX were removed and cells were remained in DMEM/FBS. Non-transfected HEK-293 cells were grown in 75-cm^2 flasks in DMEM containing 10 % FBS and antibiotics. Transfected HEK-293 cells were maintained in DMEM/FBS with 250 μg/mL geneticin and compared with non-transfected HEK-293 cells. All types of cells were maintained at 37 °C in an atmosphere containing 5 % CO_2 and 100 % humidity. Cell culture reagents, FBS, CS, and Trypsin-EDTA were from Gibco/BRL (Grand Island, NY).

Production and purification of recombinant agouti polypeptide: A 614 bp XbaI/PstI fragment of the full-length mouse agouti cDNA (10, 11) or human agouti cDNA (18) was subcloned into a XbaI/PstI digested baculoviral expression vector pAcMP3 (PharMingen, San Diego, CA). Virus incorporating this vector were produced by standard methods (14). Spodoptera frugiperda cells (Sf-9) were propagated in Grace's supplemented medium containing 10 % CS and 0.1 % pluronic F-68 and used to produce high-titer viral stocks. Fifteen liter scale production runs of murine or human agouti were produced by using *Trichoplusia ni* (*T. ni*) cells adapted to suspension (JRH Biosciences, Woodland, CA). *T. ni* cells were infected 24 hr post-seeding at a density of 1 x 10^6 cells/mL at a multiplicity of infection of 1. Conditioned media from infected cells were harvested 48 hr after infection, filtered through a Whatman 3 filter, and purified as recently described (19).

Transfection with receptor genes: The coding region of the human melanocortin 1, 3 and 5 receptors (hMC1-R, hMC3-R and hMC5-R) genes were amplified by polymerase chain reaction (PCR) and subcloned into the vector pMT4 and sequence was verified.

HEK-293 cells which appear to lack endogenous melanocortin receptors (14) were co-transfected with pMT4 and pRSV-Neo by either calcium phosphate co-precipitation method or the use of Transfectam Reagent (Promega). Clones were selected using 600 μg geneticin/mL culture media. Using ^{125}I-labeled Nle^4-D-Phe^7-α-MSH (NDP-MSH) (Bioscience Inc. King of Prussia, PA), the K_i for the clones ranged from 1.1×10^{-10} M to 5.8×10^{-10} M. Of these clones the receptor number per cell was determined for MC1-R clone, and demonstrated to be approximately 18,000 receptors per cell.

Measurement of $[Ca^{2+}]i$: $[Ca^{2+}]i$ was measured in L6, A7r5 and HEK-293 cells as previously described (17), and in 3T3-L1 cells as described by Klip et al. (20). Monolayers of non-fused L6 cells and confluent A7r5 cells after 14-16 hr starvation in a serum-free culture media were rinsed with Hank's solution (Gibco/BRL, Grand Island, NY) and detached by incubation in 2 mL of trypsin (0.05%) for 2-3 min at 37 °C and released by pipetting with culture media containing FBS. Confluent HEK-293 cells after 3-4 hr starvation in a serum-free culture media were released just by pipetting without trypsinization. Cells were harvested by centrifugation at 50 x g, and resuspended in 1 mL of HEPES-buffered salt solution (HBSS) (17) at a density of approximately 10^6 cells/mL. For 3T3-L1 cells, monolayers of differentiated cells were rinsed with serum-free DMEM and exposed to 0.05 % trypsin for 3 min. The solution was decanted, and the cells were incubated for 3 min in culture media. Cells were detached by pipetting and collected by centrifugation at 50 x g. Cells were then incubated in culture media to recover for 1 hr followed by incubation in serum-free DMEM for 4 hr at 37 °C in an atmosphere of 5 % CO_2, and collected by centrifugation and resuspended in HBSS at a density of

approximately 10^6 cells/mL. Cell suspensions were chilled in ice for 5 min and loaded with 10 μM fura-2/AM (final concentration) (Sigma, St. Louis, MO) in the dark for 20 min at 37 °C. For 3T3-L1 adipocytes chilling step was skipped. Extracellular fura-2/AM was washed three times with HBSS by centrifugation. For the $[Ca^{2+}]i$ measurements L6, A7r5, HEK-293 cells and 3T3-L1 cells were resuspended in 1 mL of HBSS and transferred to a 1-mL, 37 °C cuvette. $[Ca^{2+}]i$ levels were then measured fluorometrically in suspensions using dual excitation (340 and 380 nm)/single emission (510 nm) fluorometry as previously described (17) and $[Ca^{2+}]i$ was then calculated by the computer in the fluorometer using the equation of Grynkiewicz et al. (21). $[Ca^{2+}]i$ response to the recombinant agouti protein or to NDP-MSH was evaluated after establishing a stable baseline for each cell suspension. In experiments with Ca^{2+} channel blocker, after loading fura-2/AM cells were pretreated with nitrendipine (RBI, Natick, MA) (30 μM, final concentration) for 10 min at 37 °C prior to adding agonists.

Statistical analysis: All data were analyzed in the student's *t* test or, where appropriate, analysis of variance. Data were evaluated for equality of variance and normality of distribution prior to statistical analyses were confirmed. Data were reported as mean ± SE.

D. Results

Purified recombinant murine agouti protein induced a slow, sustained increase in $[Ca^{2+}]i$ ($p < 0.01$ vs. baseline) in L6 skeletal myocytes and A7r5 vascular smooth muscle cells (Fig. 4). Murine agouti also produced a slow, sustained increase in $[Ca^{2+}]i$ in fully differentiated 3T3-L1 adipocytes. For example, 50 nM agouti increased $[Ca^{2+}]i$ by 44 ± 4

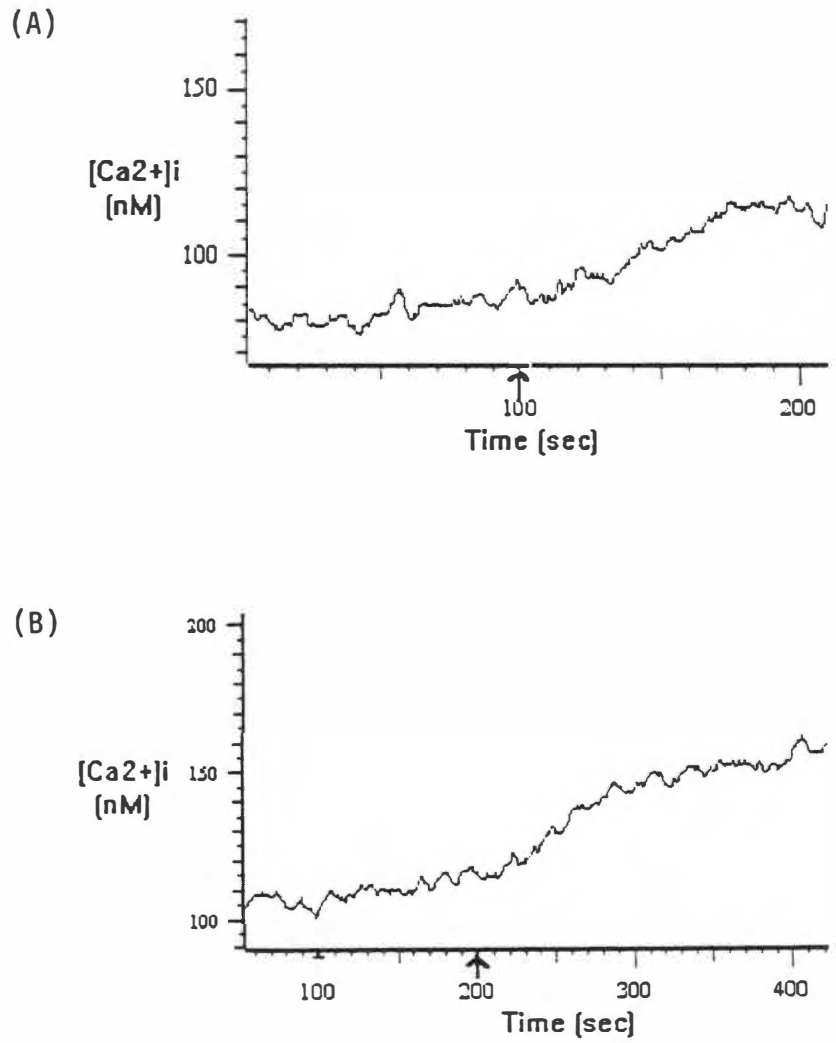

Figure 4. Effects of recombinant murine agouti protein (50 nM) on intracellular Ca^{2+} levels ($[Ca^{2+}]i$) in cultured (A) L6 skeletal myocytes and (B) A7r5 vascular smooth muscle cells. $[Ca^{2+}]i$ levels were measured as described in materials and methods. Agouti was added at times designated by the arrows.

nM over the baseline at 200 sec following agouti addition and reached at plateau of 73 ± 6 nM increase at 400 sec following agouti addition ($p < 0.03$ vs. vehicle and time controls). These data are summarized in Table 3.

The agouti effect on $[Ca^{2+}]i$ was further characterized in L6 skeletal myocytes. The $[Ca^{2+}]i$ dose-response to agouti is shown in Fig. 5; agouti stimulated $[Ca^{2+}]i$ responses with an apparent EC_{50} of 62 ± 19 nM. Ca^{2+} channel blockade with 30 μM nitrendipine reduced the response to 50 nM agouti in these cells by approximately 50 % ($p < 0.01$), from 49 ± 8 to 22 ± 6 nM.

Agouti regulates coat color via antagonism of α-MSH binding to the α-MSH receptor (MC1-R). Since melanocortin peptides also stimulate $[Ca^{2+}]i$ increases, we wanted to evaluate whether the effect of agouti on $[Ca^{2+}]i$ was also mediated through melanocortin receptors; we first measured the $[Ca^{2+}]i$ response to NDP-MSH, a stable and potent analogue of α-MSH, in HEK-293 cells stably transfected with hMC1-R, hMC3-R, hMC5-R and in non-transfected HEK-293 cells. NDP-MSH (1-10 nM) induced significant transient elevations in $[Ca^{2+}]i$ in HEK-293 cells transfected with hMC1-R, hMC3-R and hMC5-R ($p < 0.01$ vs. baseline, Fig. 6), but not in non-transfected HEK-293 cells (data not shown). Also, NDP-MSH in concentrations as high as 500 nM failed to stimulate an increase in $[Ca^{2+}]i$ in L6 or A7r5 cells (data not shown). Thus, as previously described, melanocortin peptide is capable of stimulating transient increases in $[Ca^{2+}]i$ in a melanocortin receptor-dependent manner.

We then examined what agouti's effect on $[Ca^{2+}]i$ would be in these melanocortin receptor transfected cell lines. As shown in Table 4, agouti (20 nM) provoked an increase

Table 3. Effects of purified recombinant murine agouti protein on $[Ca^{2+}]i$ in L6 skeletal myocytes, A7r5 vascular smooth muscle cells (VSMCs) and 3T3-L1 adipocytes[1]

	$[Ca^{2+}]i$ Increase over Baseline (nM)	
	25 nM Agouti	50 nM Agouti
L6 Myocytes	32 ± 1[a]	49 ± 8[a, b]
A7r5 VSMCs	46 ± 9[a]	74 ± 6[a, b]
3T3-L1 Adipocytes	33 ± 6[a]	73 ± 6[a, b]

[1]Data shown are at 400 sec following agouti addition.

[a]$p < 0.03$ vs. vehicle control

[b]$p < 0.03$ vs. 25 nM agouti

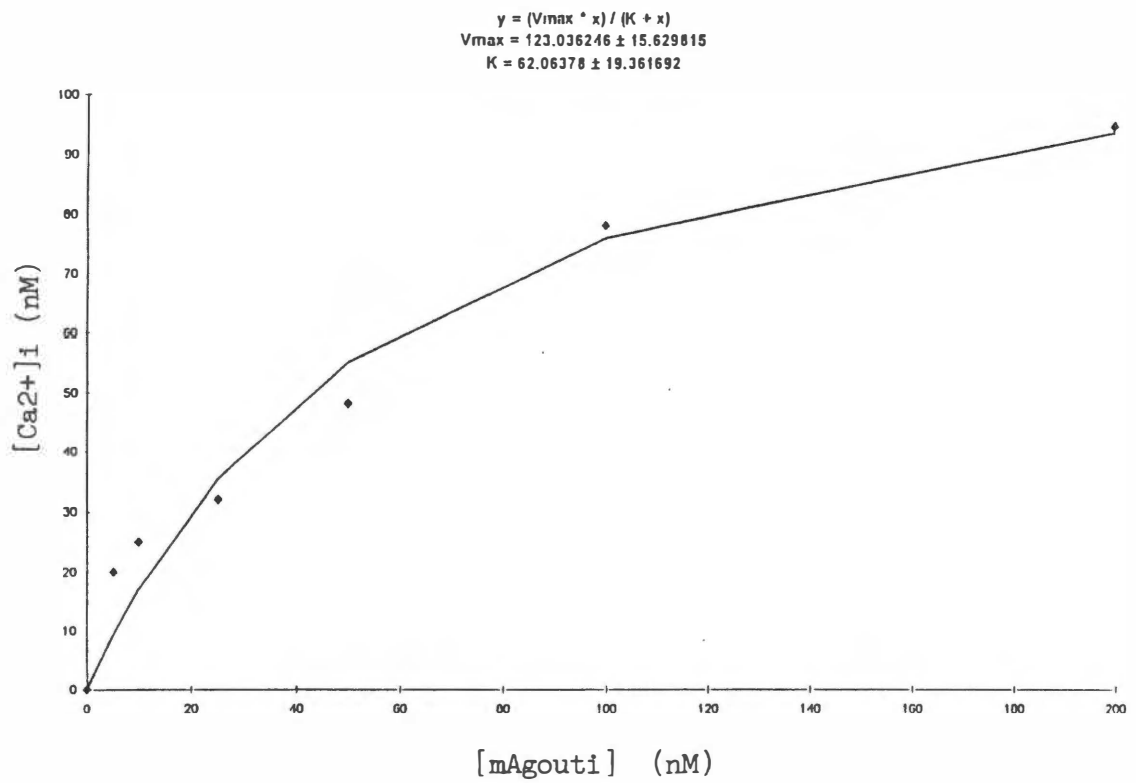

Figure 5. Dose-response curve of effects of recombinant murine agouti protein (mAgouti) (nM) on intracellular Ca^{2+} levels ($[Ca^{2+}]i$) in cultured L6 skeletal myocytes. $[Ca^{2+}]i$ responses were measured as described in materials and methods, and values reported are for the response plateau at 400 sec following agouti addition. The data were fit by non-linear least squares analysis, yielding an half-maximal concentration (EC_{50}) of 62 ± 19.

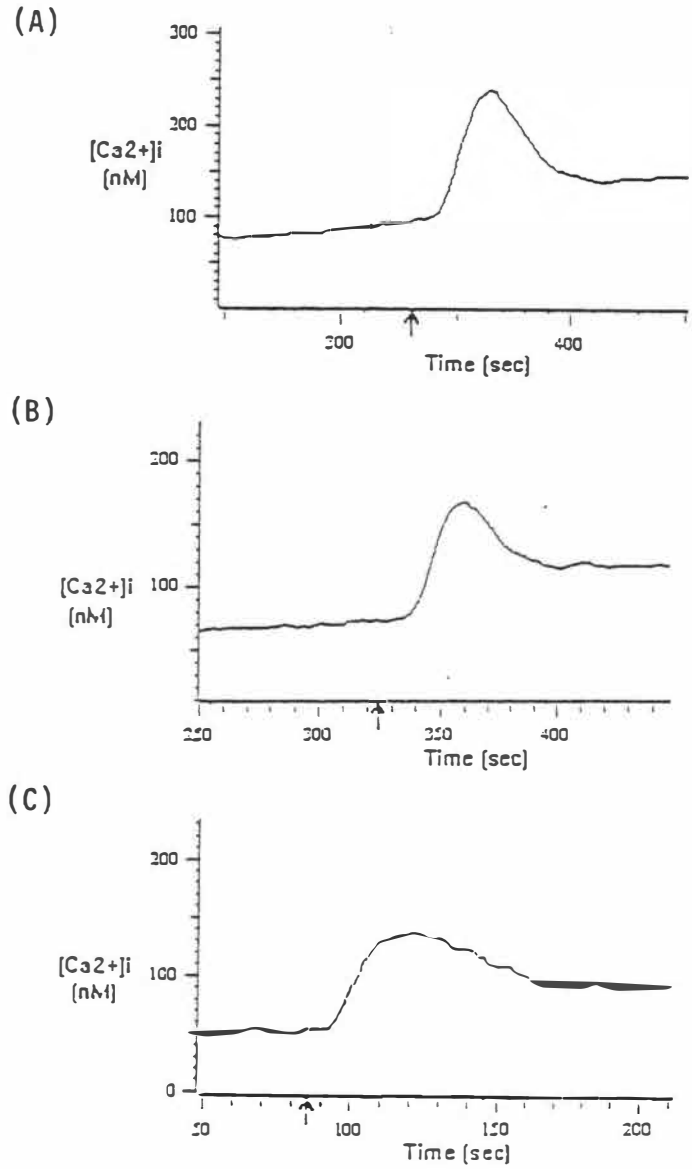

Figure 6. Effects of NDP-MSH (1-10 nM) on intracellular Ca^{2+} levels ($[Ca^{2+}]i$) in human embryonic kidney 293 cells transfected with (A) hMC1-R (1 nM), (B) hMC3-R (10 nM), or (C) hMC5-R (10 nM). $[Ca^{2+}]i$ levels were measured as described in materials and methods. NDP-MSH was added at times designated by the arrows.

Table 4. Effects of purified recombinant human agouti protein on $[Ca^{2+}]i$ in human embryonic kidney 293 (HEK-293) cells transfected with melanocortin receptors[1]

	$[Ca^{2+}]i$ Increase over Baseline (nM)[2]
HEK-293	*
HEK-293-hMC1R[3]	57 ± 9[a]
HEK-293-hMC3R[3]	77 ± 14[a]
HEK-293-hMC5R[3]	*

* No detectable increase

[1]Data shown are at 400 sec following agouti addition.

[2]Response to 20 nM recombinant human agouti protein

[3]hMC1R, human melanocortin 1 receptor; hMC3R, human melanocortin 3 receptor; hMC5R, human melanocortin 5 receptor.

[a]$p < 0.01$ vs. baseline

in $[Ca^{2+}]i$ over the baseline in the hMC1-R and hMC3-R HEK-293 cell lines, while no response was observed in either the hMC5-R or non-transfected HEK-293 cells.

Further characterization of this response was performed by using a dose response of recombinant human agouti protein on $[Ca^{2+}]i$ in the hMC1-R line (Fig. 7). Agouti stimulated $[Ca^{2+}]i$ with an approximate EC_{50} of 18 ± 0.07 nM.

E. Discussion

Data from the present study directly demonstrate that recombinant murine agouti protein elicits significant increases in $[Ca^{2+}]i$ in several distinct cell types, including L6 skeletal myocytes, A7r5 vascular smooth muscle cells and 3T3-L1 adipocytes. These agouti effect on $[Ca^{2+}]i$ were manifested as a gradual, sustained increase rather than a rapid transient, and is in part dependent upon Ca^{2+} channel mediated Ca^{2+} influx, consistent with our recent report of increased skeletal muscle Ca^{2+} influx in A^{vy} mice (17).

This is also consistent with other Ca^{2+} agonists which inhibit insulin signaling. For example, Kelly et al. (22) reported that treatment of adipocytes with agonists which either produced sustained increases in $[Ca^{2+}]i$ or biphasic responses with a sustained component, such as norepinephrine or KCl, resulted in significant inhibition of insulin-stimulated glucose transport. In contrast, oxytocin in concentrations sufficient to cause 3-5 fold increases in $[Ca^{2+}]i$ which were transient rather than sustained, similar to those found with NDP-MSH in the present study, were without effect on insulin action. Moreover, an additional common feature of those agonists which were able to inhibit insulin signaling was that they promoted Ca^{2+} influx, and there was a high degree of correlation ($r = 0.92$) between the sustained $[Ca^{2+}]i$ response to these agonists and the degree of inhibition of

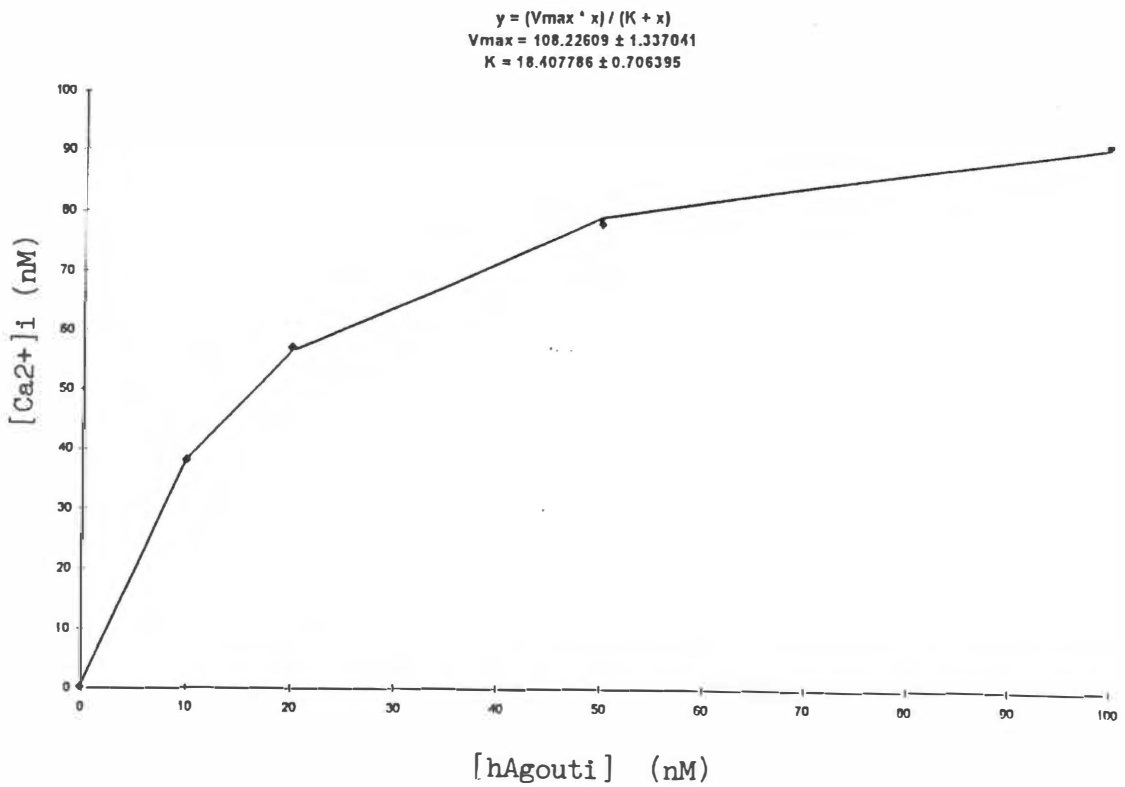

Figure 7. Dose response curve of the effects of recombinant human agouti protein (hAgouti) (nM) on intracellular Ca^{2+} levels ($[Ca^{2+}]i$) in human embryonic kidney 293 cells transfected with hMC1-R. $[Ca^{2+}]i$ responses were measured as described in materials and methods, and values reported are for the response plateau at 400 sec following agouti addition. The data were fit by non-linear least squares analysis assuming a single binding site, yielding an half-maximal concentration (EC_{50}) of 18 ± 0.07.

insulin action. It was suggested that this correlation was related to Ca^{2+} channel opening rather than to the actual increase in $[Ca^{2+}]i$, as ionomycin caused a sustained elevation in $[Ca^{2+}]i$ which was derived from intracellular stores (22). Accordingly, net elevations in $[Ca^{2+}]i$ secondary to sustained agouti exposure may maintain a chronic excess in Ca^{2+} channel-mediated Ca^{2+} influx and thereby induce insulin resistance in A^{vy} mice.

Agouti-induced increases in $[Ca^{2+}]i$ in multiple cell types may result in tissue-specific consequences. For example, since increased $[Ca^{2+}]i$ results in diminished insulin signaling (1, 3), as discussed above, agouti-mediated elevations in $[Ca^{2+}]i$ are predicted to result in insulin resistance in skeletal myocytes and adipocytes. In vascular smooth muscle cells, Ca^{2+} plays a central role in both regulation of contraction and proliferation (3). Consequently, agouti-induced increases in vascular smooth muscle $[Ca^{2+}]i$ (as typified by A7r5 cells) may contribute to the hypertension which is characteristic of obese, insulin-resistant states. Indeed, our preliminary data indicate that vascular rings isolated from A^{vy} mice exhibit exaggerated contractile responses to presser agonists (unpublished observation).

In addition to contributing to insulin resistance, increased $[Ca^{2+}]i$ in adipocytes appears to stimulate lipogenesis. We have recently reported that incubation of 3T3-L1 adipocytes in an agouti-containing medium upregulates fatty acid synthase (FAS) mRNA levels and activity (23). Moreover, these effects were reversed by Ca^{2+} channel blockade with nitrendipine and could be replicated by depolarization with 50 mM KCl (24). Thus, agouti-induced increases in $[Ca^{2+}]i$ appear to stimulate *de novo* lipogenesis and may thereby contribute to the obesity of A^{vy} mice. This phenomenon may be relevant to human

obesity as well, as we have also found recombinant human agouti protein to stimulate an increase in $[Ca^{2+}]i$ in freshly isolated human adipocytes (unpublished observation).

Although agouti regulation of $[Ca^{2+}]i$ appears to be primarily via Ca^{2+} channels, the mechanism of this Ca^{2+} regulation is unknown. However, agouti has recently been demonstrated to have significant spatial cysteine homology to both ω-conotoxins and plectoxins, both of which have high affinity for neuronal Ca^{2+} channels (25). The action of ω-conotoxin GVIA, one of the ω-conotoxins from the venom of the marine snail *Conus geogprhus*, appears to be through direct interaction of the toxin with a receptor closely linked to an N-type Ca^{2+} channel, without a second messenger, followed by a very slow dissociation (26). Plectoxins, contained in venom produced by the spider *Plectreurys tristis*, inhibit N-type Ca^{2+} channels as well as unclassified Ca^{2+} channels, although the underlying mechanisms are unclear (27). The structural similarity of agouti protein with these two toxins suggests that agouti may represent a class of molecules related to the toxins that interact with a specific Ca^{2+} channel subtype or as of yet undefined Ca^{2+} channel (25). Consistent with this, we have reported that agouti-induced increases in $[Ca^{2+}]i$ are abolished in the absence of extracellular Ca^{2+} (17) and have also demonstrated inhibition by Ca^{2+} channel blockade with nitrendipine in the present study.

Agouti regulation of $[Ca^{2+}]i$ may also depend upon an interaction with melanocortin receptors in HEK-293 cells. Several studies have demonstrated that melanocortin action is linked to $[Ca^{2+}]i$ as well as cAMP signaling (28, 29). For example, adrenocorticotropic hormone (ACTH) binding to its receptor in adipocytes stimulates both adenylate cyclase and increased $[Ca^{2+}]i$, and both are required for ACTH-induced

lipolysis (28). Moreover, ω-conotoxin inhibits Ca^{2+} channels in ACTH target tissues, diminishing the biological effects of the hormone in several tissues, including adrenal glomerulosa cells (30), lymphocytes (31) and neuronal cells (32). Consistent with this, all melanocortin receptor transfected cells in the present study exhibited NDP-MSH stimulated increases in $[Ca^{2+}]i$, and these cells also respond to α-MSH with an increase in cAMP (14). However, co-administration of agouti failed to inhibit the effects of NDP-MSH on $[Ca^{2+}]i$ in HEK-293 cells transfected with melanocortin receptors (data not shown), similar to the previous report in which agouti did not antagonize cAMP production in hMC3-R or hMC5-R transfected cells (14), suggesting that MC3-R and MC5-R do not appear to be direct targets for agouti. In contrast, non-transfected HEK-293 cells failed to respond to agouti, while HEK-293 cells transfected with either the hMC1-R or hMC3-R exhibited significant $[Ca^{2+}]i$ responses. These data indicate that agouti regulation of $[Ca^{2+}]i$ in HEK-293 cells is dependent upon an interaction with melanocortin receptors. However, this interaction is clearly different from agouti regulation of coat color, which is dependent upon a competitive antagonism with α-MSH (14). However, the role of melanocortin receptors in agouti-mediated Ca^{2+} signaling in other cell types requires further clarification. The only melanocortin receptor reported to be expressed in muscle cells is MC5-R (33). Consistent with this, NDP-MSH failed to elicit an $[Ca^{2+}]i$ response in either type of muscle cell (L6 and A7r5) examined in the present study. However, in the HEK-293 cells, presence of MC5-R was not sufficient to permit agouti regulation of $[Ca^{2+}]i$, while MC1-R and MC3-R were sufficient. Thus, it is not yet clear whether Ca^{2+} signaling in muscle cells is dependent upon the presence of

melanocortin receptors. Nonetheless, we have demonstrated that Ca^{2+} signaling in these cells is also not mediated by melanocortin receptor antagonism.

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III. THE EFFECTS OF CALCIUM CHANNEL BLOCKADE ON AGOUTI-INDUCED OBESITY[1]

A. Abstract

We previously observed that obese viable yellow (A^{vy}) mice exhibited increased intracellular Ca^{2+} ($[Ca^{2+}]i$) and fatty acid synthase (FAS) gene expression. Further, recombinant agouti protein increased both in cultured adipocytes, and these effects were inhibited by Ca^{2+} channel blockade. Accordingly, we determined the effect of Ca^{2+} channel blockade with nifedipine (1 g/kg diet for 30 days) on FAS and obesity in transgenic mice expressing the agouti gene in a ubiquitous manner. The transgenic mice were initially significantly heavier (30.54 ± 0.66 vs. 27.26 ± 0.28 g; $p < 0.001$) and exhibited a 0.81 °C lower initial core temperature ($p < 0.0005$) and an approximately 2-fold increase in fat pad weights ($p = 0.002$), 7-fold increase in adipose FAS activity ($p = 0.009$), and 2-fold increase in plasma insulin level ($p < 0.05$) compared to control mice. Nifedipine treatment resulted in an 16 % decrease in fat pad weights ($p < 0.007$) and a 74 % decrease in adipose FAS activity ($p = 0.03$), normalized circulating insulin levels and insulin-sensitivity ($p < 0.05$), and transiently elevated core temperature in the transgenic mice, but was without effects in the control mice. These data suggest that agouti regulates FAS, fat storage and possibly thermogenesis, at least partially, via a $[Ca^{2+}]i$ dependent mechanism, and that Ca^{2+} channel blockade may partially attenuate agouti-induced obesity.

[1]This manuscript has been submitted for publication in similar form with co-authors Mynatt, R. L., Moore, J. W., Woychik, R. P., Moustaid, N., and Zemel, M. B. in: FASEB J.

B. Introduction

Mice with dominant mutations at the agouti locus, including lethal yellow (A^{y}) and viable yellow (A^{vy}) are characterized by obesity, insulin resistance, and yellow coat color (1, 2). The mouse agouti gene normally regulates differential pigment production in hair-bulb melanocytes (3). Each melanocyte in the hair bulb switches between eumelanin (black) and phaeomelanin (yellow), producing the wild-type mouse coat color of a black hair with a subapical band of yellow (3). Agouti mutations disrupt this switching process and form a dominance hierarchy in which the dominant alleles produce a yellow coat and the recessive alleles produce a black coat (3).

Although agouti is normally expressed in neonatal skin, dominant agouti mutations are characterized by ectopic overexpression of agouti through out life (2, 4, 5). This is due to mutations in the promoter/regulatory region rather than in protein-coding domain. For example, the A^{y} mutation exhibits a structural deletion corresponding to all but the promoter and 5′-noncoding first exon of an another gene, *Raly*, which is tightly linked to agouti. This results in overexpression of agouti in a ubiquitous manner under the control of the *Raly* promoter (6). Unlike A^{y}, the A^{vy} mutation contains an insertion of an intracisternal A particle element (IAP), which activates agouti expression by initiating transcription from a cryptic promoter found within the IAP long terminal repeats (7). In both cases, however, the result is ubiquitous expression of agouti mRNA.

Transgenic mice designed to express the agouti coding portion in a ubiquitous manner also develop a syndrome of obesity, hyperinsulinemia, hyperglycemia and yellow

coat color, similar to A^{vy} mutation, demonstrating that ectopic overexpression of the agouti gene is directly responsible for pleiotropic effects associated with dominant agouti mutations (8, 9). However, the mechanism linking this pigmentation gene to obesity has not yet been identified.

Several lines of evidence indicated that intracellular Ca^{2+} ($[Ca^{2+}]i$) appears to be involved in metabolic derangements, including obesity and insulin resistance (10-12). Obese patients exhibited increased basal $[Ca^{2+}]i$ in adipocytes (10), while increasing $[Ca^{2+}]i$ in rat adipocytes reduced insulin stimulated glucose transport (11). Further, Ca^{2+} channel blockade enhanced insulin sensitivity in obese and glucose intolerant subjects (12).

We have recently reported an increase in the expression of fatty acid synthase (FAS), a key enzyme in *de novo* fatty acid synthesis, in A^{vy}/a mice compared to lean controls (13). We have also reported that A^{vy}/a mice exhibited an elevation both in steady state $[Ca^{2+}]i$ and in Ca^{2+} influx rate (14). This increase in $[Ca^{2+}]i$ was closely correlated with both the degree of ectopic expression of the agouti gene and body weight (14), suggesting the possibility of a causal relationship between $[Ca^{2+}]i$ and obesity mechanism in these animals. Furthermore, we have also found recombinant agouti protein to stimulate both mRNA levels and activity of FAS and to increase triglyceride accumulation in 3T3-L1 adipocytes. Moreover, agouti-mediated stimulation of FAS activity was completely prevented by Ca^{2+} channel blockade (13). Furthermore, agouti-stimulation of FAS was mimicked by stimulation of Ca^{2+} influx in 3T3-L1 adipocytes with other Ca^{2+} agonists, such as KCl or parathyroid hormone (15), suggesting that $[Ca^{2+}]i$ modulation may be an important means of regulating FAS.

Accordingly, Ca^{2+} channel antagonism in animals overexpressing the agouti gene may be predicted to inhibit *de novo* lipogenesis and thereby attenuate the obesity characteristic of these animals. The present study was conducted to evaluate this possibility, and we demonstrate herein that Ca^{2+} channel blockade reduces FAS mRNA levels and activity and decreases adipose tissue mass in the transgenic mice which ubiquitously overexpress agouti.

C. Material and Methods

Animals and diets: Transgenic mice were generated as previously described (8). In short, transgenic mice were made by pronuclear microinjection of a β-actin promoter-agouti transgene into FVB/N single cell embryos and maintained on the FVB/N background. Prior to being placed on the experimental diets, 7-week-old male transgenic and control mice were acclimatized on a powdered diet (Mouse Diet 5015, PMI Feeds, ≥ 11 % fat w/w) for 1 week. They were then randomly assigned to either a control or nifedipine (Sigma, St. Louis, MO) (1g/kg diet) diet and were fed *ad libitum* for 30 days. On day 27, food was held overnight for 12 hr fasting and blood was collected from the tail vein for glucose determination, followed by refeeding. On day 30, animals were anesthetized with sodium pentobarbital (50 mg/kg body weight) (Abbott Lab. North Chicago, IL) and blood was obtained by cardiac puncture for blood glucose and plasma triglyceride and insulin determination. Fat pads (epididymal, perirenal, retroperitoneal, inguinal, and subscapular fats) and skeletal muscles (gastrocnemius muscles) were dissected, immediately weighed, frozen in liquid nitrogen, and stored at -80 °C. FAS activity and mRNA levels were measured in adipose tissues as described below.

Core temperature: Core temperature was used as an indirect metabolic index to determine if (a) thermogenesis is decreased in transgenic mice overexpressing agouti, and (b) if nifedipine treatment increases core temperature. Temperature was measured weekly via a thermocouple (Columbus Instr. Columbus, OH). The probe was inserted a constant distance (1.8 cms) into the rectum of animal. After stabilization (10 sec), the temperature was recorded every 5 sec for 30 sec. All temperature measurements were made between 9:00-10:30 A.M..

Blood glucose and plasma insulin and triglyceride analysis: Fasted and fed Blood glucose was measured using blood glucose monitoring system (Milpitas, CA). Plasma triglyceride levels were measured spectrophotometrically using an enzyme-based assay kit (Sigma, St. Louis, MO), and plasma insulin levels were measured by radioimmunoassay kit (INCSTAR, Stillwater, MN).

Fatty acid synthase activity: Fatty acid synthase activity was measured by a modification of the spectrophotometric method (16). Subcutaneous adipose tissue were sonicated (1:3 w/v) in 250 mM sucrose buffer containing 1 mM EDTA (Gibco, Gaithersburg, MD), 1 mM dithiothreitol and 100 μM phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO) (pH 7.4). Homogenates were centrifuged at 14,000 x g for 15 min (at 4 °C), and the supernatants were used for enzyme assays. Assays were started by the addition of malonyl CoA, and enzyme activities were expressed as nmol NADPH oxidized/min/mg of protein. Protein was determined by the modified method of Lowry using bovine serum albumin as a standard (17).

Dot blot analysis: Total cellular RNA was extracted by cesium chloride density gradient method (13). cDNA probes for FAS (pFAS7, cloned by Dr. J. W. Porter's group and kindly provided by Dr. A. G. Goodridge) were radiolabeled by the random primer method. Dot blot analysis using 1, 3, and 5 μg RNA was performed by a modification of the method of Meyuhas et al. (18). Total RNA was placed in eppendorf tube, brought to 50 μl with ice-cold water, added 30 μl 20 x SSC and 20 μl 37 % formaldehyde, and incubated at 65 °C for 10 min. Samples were spotted into nylon membranes (NEN, Wilmington, DE) in a vacuum dot-blot apparatus, rinsed with 4 x SSC, and air dried. The membranes were hybridized with radiolabeled FAS probe as described previously (19). Following visualization and quantitation, the membranes were stripped and reprobed with β-actin as a loading control, and data are expressed as FAS:actin ratio. Visualization and quantitation were conducted using Ambis 4000 direct β-counting and imaging system.

Statistical analysis: All data were presented as mean ± standard error for four groups of mice; control mice on control diet, control mice on nifedipine diet, transgenic mice on control diet, and transgenic mice on nifedipine diet. Data were analyzed via two-way (diet x animal) analysis of variance or, in cases where only two groups were being compared, by student *t* test.

D. Results

At study initiation (8 weeks of age), the transgenic mice (n = 12) exhibited significantly higher body weight than the control mice (n = 10) (30.54 ± 0.66 vs. 27.26 ± 0.28 g; $p < 0.001$). The increased body weight of the transgenic mice was maintained over the 30 days on diet, and nifedipine was without effect in both groups (Fig. 8). The

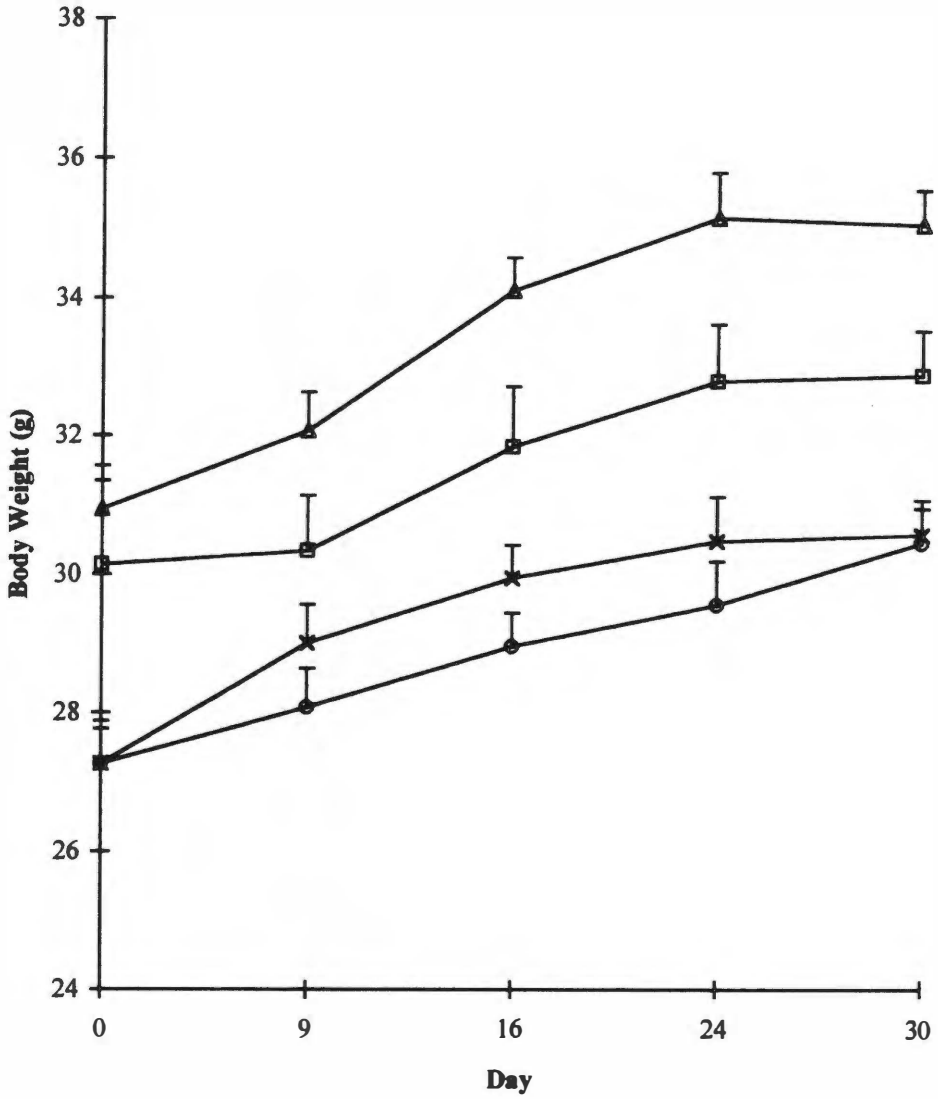

Figure 8. Weight gain of transgenic (Tg/-) and control (+/+) mice in either control or nifedipine diet. Average weights in grams are plotted over the 30 days of experiment. Data are reported as mean ± SE. o; control mice on control diet (n = 5), ×; control mice on nifedipine diet (n = 5), □; transgenic mice on control diet (n = 6), Δ; transgenic mice on nifedipine diet (n = 6)

transgenic mice also exhibited an approximately 2-fold increase in fat pad mass (both visceral and subcutaneous) compared to the control mice (2.62 ± 0.27 vs. 1.21 ± 0.19 g; p = 0.002). However, nifedipine treatment resulted in a significant reduction in fat pad weight, compared to control diet (1.81 ± 0.14 vs. 1.58 ± 0.23 g, $p < 0.001$ in visceral [epidydimal, perirenal and retroperitoneal] fat; 0.81 ± 0.14 vs. 0.62 ± 0.1 g, p = 0.02 in subcutaneous [inguinal and subscapular] fat) in the transgenic mice, but was without effect in the control mice. The total weight of the five fat pads measured was decreased by 16 % in the nifedipine-treated transgenic animals ($p < 0.007$). Fat pad weight expressed as % of body weight is shown in Fig. 9. Gastrocnemius muscle (an indicator of skeletal muscle mass) weight was not different between control and transgenic mice (data not shown); however, nifedipine treatment significantly increased gastrocnemius muscle weight by 11 % compared to the control diet (0.284 ± 0.005 vs. 0.253 ± 0.005 g; p = 0.0009) in the transgenic mice, and was without effect in the control mice. When expressed as % of body weight, gastrocnemius muscle weight follows similar trends, as shown in Fig. 10. This combination of reduced fat pad mass and increased muscle mass may explain the lack of nifedipine effect on body weight in the transgenic mice.

At 8 weeks of age, the transgenic mice had a 0.81 °C lower core temperature than the control mice (Fig. 11), indicating decreased thermogenesis in these animals. Nifedipine treatment selectively increased core temperature in the transgenic mice by 0.62 °C (38.13 ± 0.24 to 38.75 ± 0.22 °C; p = 0.02) at day 16 of treatment, but this effect did not persist until the end of the experiment.

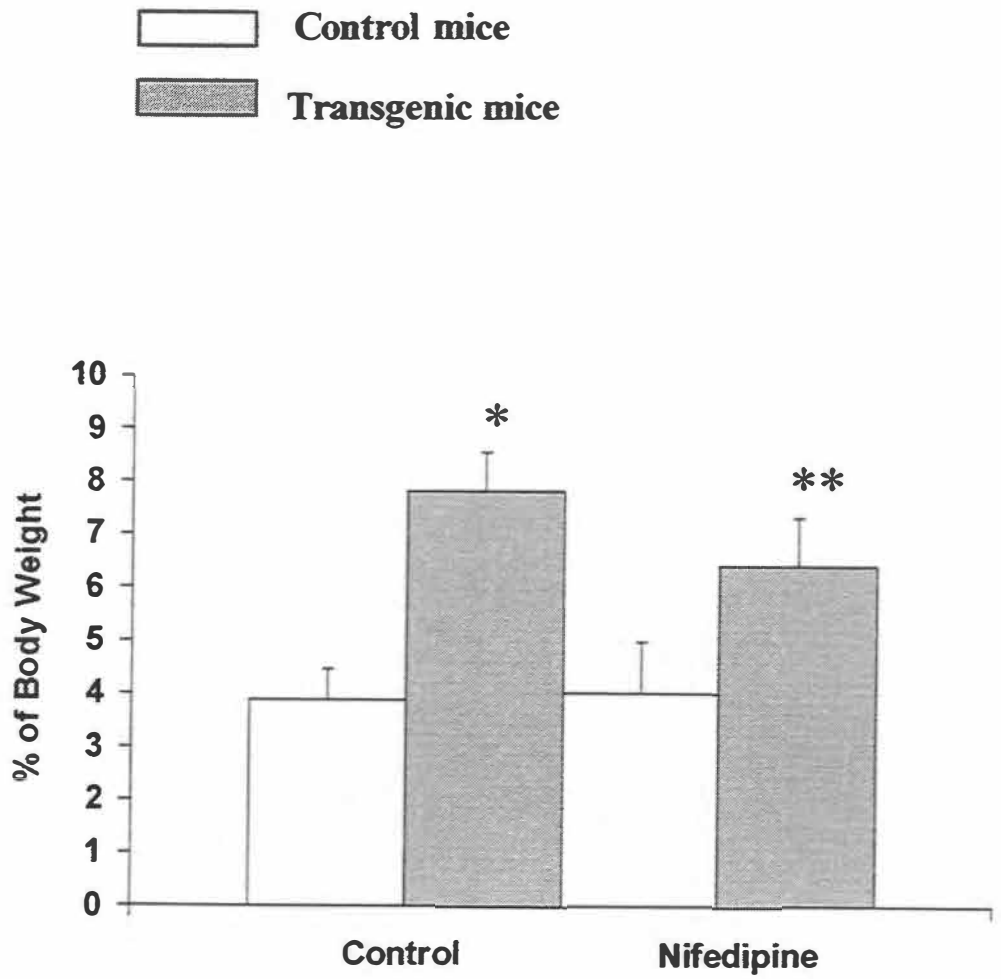

Figure 9. The effect of nifedipine treatment (30 days) on fat pad weights in transgenic (Tg/-)[1] and control (+/+)[2] mice. Average of the total amount of visceral fat pads (epididymal, perirenal, and retroperitoneal fat) and subcutaneous fat pads (inguinal and subscapular fat) is expressed as % of body weight. Data are reported as mean ± SE. *$p < 0.007$ vs. control mice on the same diet, **$p < 0.007$ vs. transgenic mice on control diet

[1]n = 5 in control diet and n = 6 in nifedipine diet, [2]n = 4 in both diets

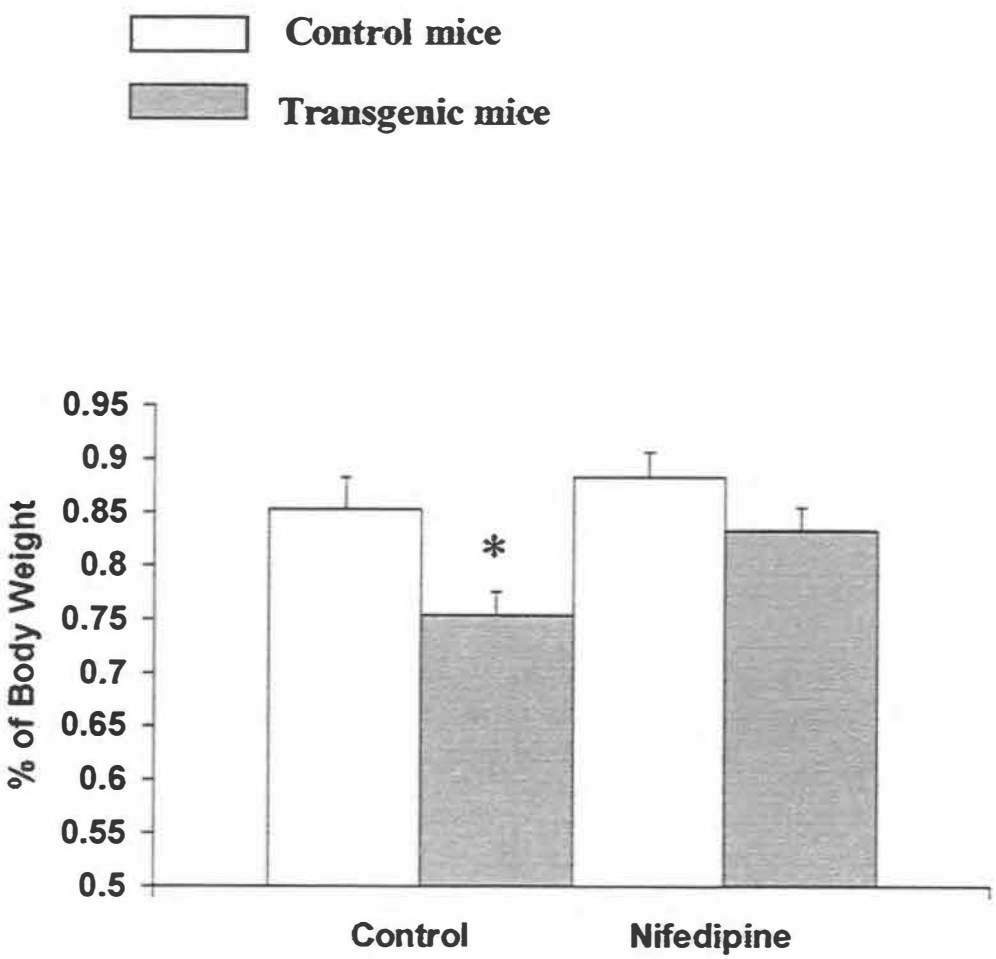

Figure 10. The effect of nifedipine treatment (30 days) on gastrocnemius skeletal muscle weight in transgenic (Tg/-)[1] and control (+/+)[2] mice. Average weight of gastrocnemius skeletal muscle is expressed as % of body weight. Data are reported as mean ± SE.

*$p < 0.02$ vs. control mice on the same diet

[1]n = 5 in control diet and n = 6 in nifedipine diet, [2]n = 4 in both diets

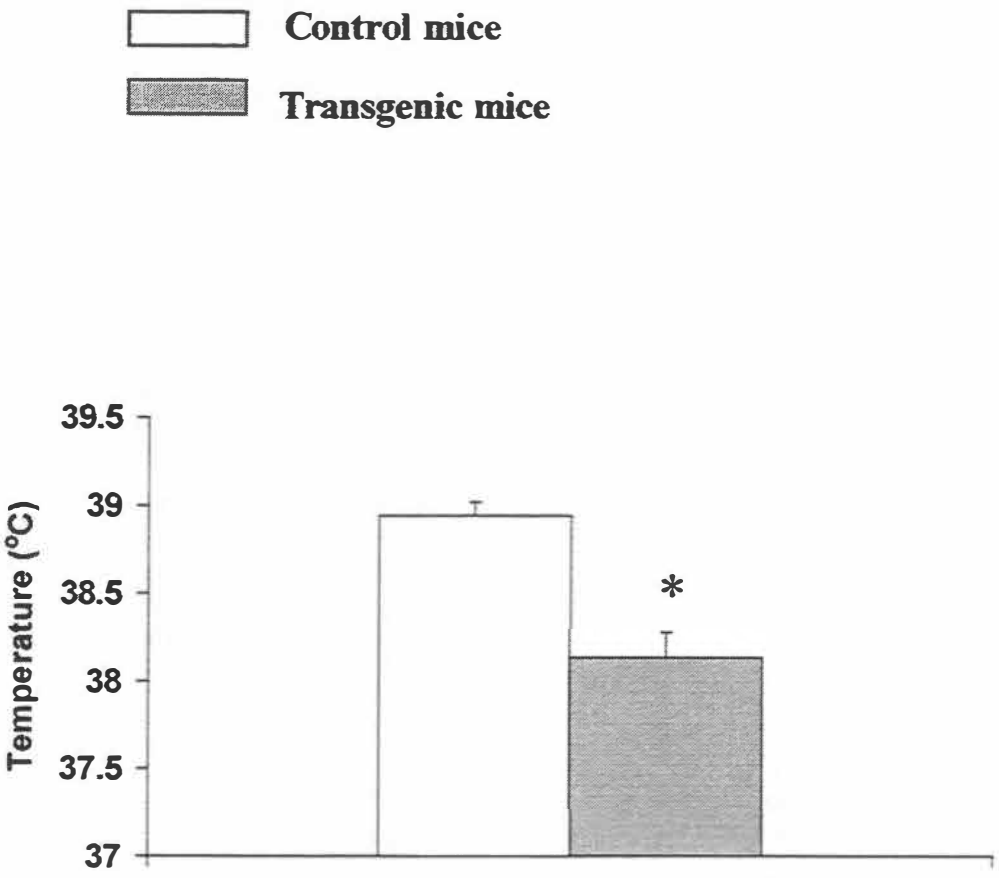

Figure 11. The basal (day 0) core temperature in transgenic (Tg/-)[1] and control (+/+)[2] mice at 8 weeks of age. Data are reported as mean ± SE. * $p < 0.0005$

[1]n = 12, [2]n =10

To test the role of *de novo* fatty acid synthesis in agouti-associated obesity, we measured the activity of FAS in adipose tissue. The transgenic mice exhibited a 7.2-fold increase in FAS activity in subcutaneous adipose tissue compared to the control mice, while nifedipine treatment completely prevented this stimulation of FAS activity (Fig. 12). Similarly, there was a modest decrease in FAS mRNA levels in visceral adipose tissue of the transgenic mice treated with nifedipine compared to that of the transgenic mice on the control diet (Fig. 13).

The transgenic mice had approximately 2-fold higher fed plasma insulin levels than the control mice ($p < 0.05$) (Table 5). Nifedipine treatment completely blocked the hyperinsulinemia in the transgenic mice, but was without effect on the control mice (Table 5). Nifedipine treatment also improved insulin sensitivity, as manifested by the fall in plasma insulin to glucose ratio in the transgenic mice ($p < 0.05$) (Table 5). Nifedipine treatment was without effect on blood glucose levels in either fasted or fed mice. There was also no effect of nifedipine on fasted plasma triglyceride contents (Table 5).

E. Discussion

Ca^{2+} channel antagonism with nifedipine resulted in a reduction of fat pad mass in the transgenic mice accompanied by an increase in skeletal muscle mass. This suggests that $[Ca^{2+}]i$ may modulate the flow of energy between storage in adipose tissue and its use for muscle accretion. This concept is supported by the observation that the reduction in core temperature in the transgenic mice was partially corrected with nifedipine treatment, although this correction was not sustained beyond two weeks. The reason for the

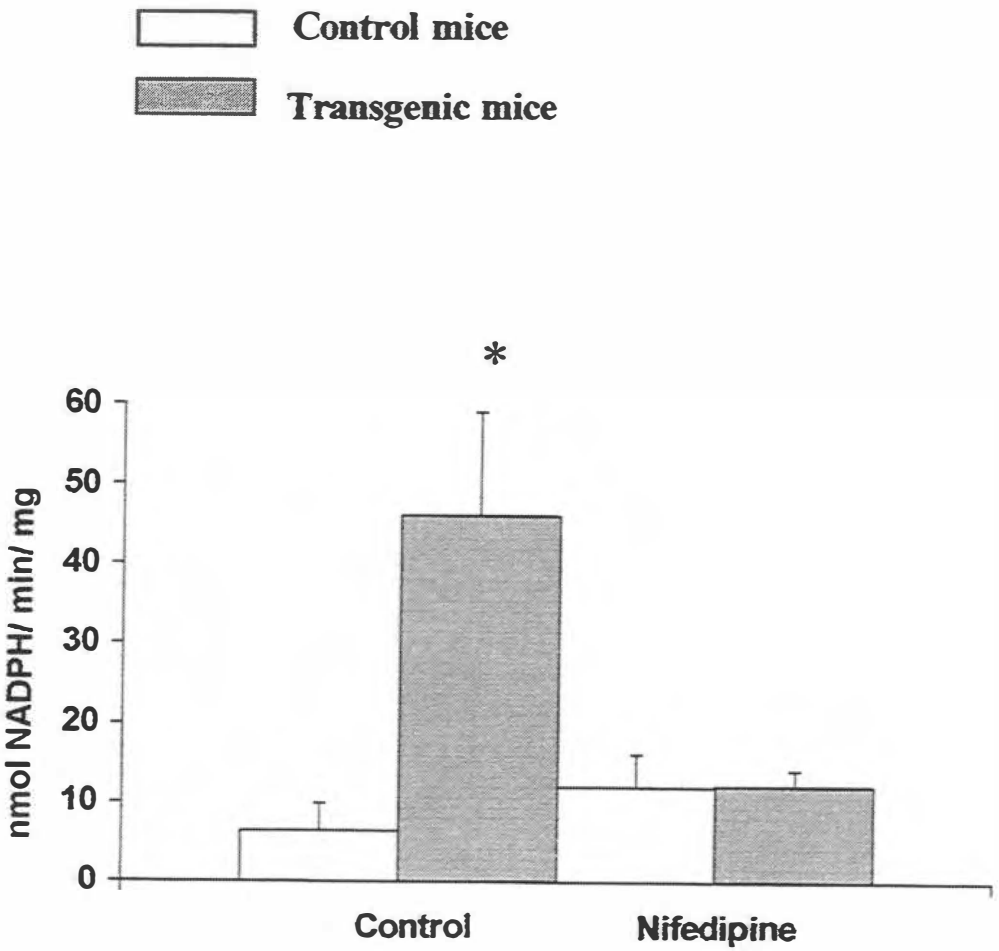

Figure 12. The effect of nifedipine treatment (30 days) on fatty acid synthase activity in subcutaneous (inguinal and subscapular) adipocytes of transgenic (Tg/-)[1] and control (+/+)[2] mice. Enzyme activity is expressed in nmol NADPH oxidized/min/mg of protein. Data are reported as mean ± SE. * $p = 0.009$ vs. control mice on the same diet

[1]n = 5 in control diet and n = 6 in nifedipine diet, [2]n = 4 in both diets

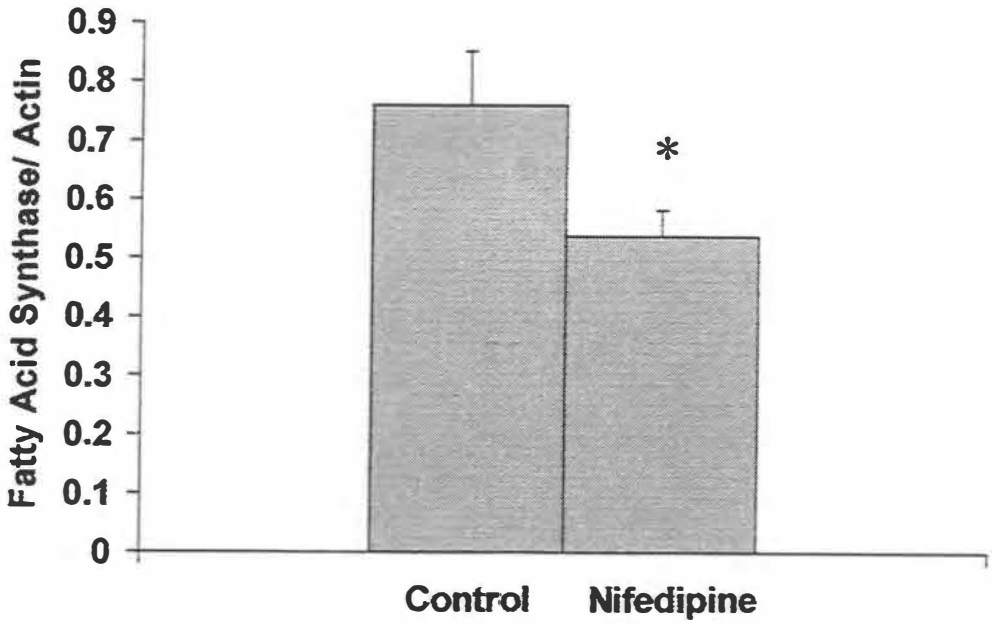

Figure 13. The effect of nifedipine treatment (30 days) on fatty acid synthase mRNA levels in visceral (epididymal, perirenal and retroperitoneal) adipocytes of transgenic (Tg/-) mice. Total RNA was extracted from visceral adipose tissue of transgenic mice either on control or nifedipine diet, and analyzed by dot blot as described in methods and materials. Data were not available from the control mice due to the limiting quantities of adipose tissue available from those lean animals. Data obtained from the direct β-counting imaging system are normalized to RNA levels for β-actin. Data are reported as mean ± SE. * $p = 0.04$, $n = 5$

Table 5. Blood glucose and plasma insulin and triglyceride levels

Animal		Control		Transgenic	
Treatment		Control[1]	Nifedipine[1]	Control[2]	Nifedipine[3]
Glucose (mg/dL)	(fasted)	121.3 ± 6.5	125.3 ± 7.8	115.5 ± 8.9	100.4 ± 15.2
	(fed)	243.0 ± 7.6	213.8 ± 18.6	230.6 ± 21.6	235.8 ± 9.7
Insulin (ng/mL)	(fed)	14.96 ± 5.3	16.74 ± 8.69	32.39 ± 4.9[a]	13.40 ± 1.96
Insulin/Glucose	(fed)	0.06 ± 0.02	0.084 ± 0.05	0.140 ± 0.02[a]	0.057 ± 0.01
Triglyceride (mg/dL)	(fasted)	107 ± 14.2	83.4 ± 20.1	76.5 ± 11.6	62.8 ± 5.6

Values are the mean ± SE. Data were compared in a row.

[1]n = 4

[2]n = 5

[3]n = 6

[a]$p < 0.05$ vs. control mice on the same diet

transient nature of this core temperature elevation is not clear, although it may be developmentally related, as the nifedipine intervention was initiated well after a stable decrease in core temperature was apparent in the transgenic animals.

FAS is a multifunctional enzyme that carries all of the 7 reactions involved in the synthesis of long chain saturated fatty acid (palmitate) from acetyl CoA and malonyl CoA (20). FAS protein concentration closely parallels its enzyme activity and is highly sensitive to nutritional and hormonal states (20-23). Fasting or high fat diet causes a dramatic suppression of FAS synthesis, while refeeding or high carbohydrate diet, especially glucose, markedly increases FAS synthesis (25-27). Several hormones, including insulin, stimulate FAS expression and activity, while glucagon and cAMP suppress them (28). An insulin response element has been in the FAS promoter region (29, 30), and chronic hyperinsulinemia induces an increase in FAS mRNA levels and activity in both rat liver and white adipose tissue (27). Moreover, obese Zucker rats have been reported to overexpress FAS mRNA in adipose tissue (31).

Nifedipine treatment normalized both FAS mRNA levels and activities and plasma insulin levels in the transgenic mice. The mechanism of the insulin lowering effect of nifedipine is not clear. However, since an increase in Ca^{2+} is a signal for β-cell insulin secretion (32), and several Ca^{2+} antagonists (such as verapamil, nifedipine, nitrendipine, and other dihydropyridines) have been found in vitro to induce a dose-related reversible inhibition of insulin release (33), it is possible that agouti-induced Ca^{2+} influx may stimulate β-cell insulin release, and that this effect is blocked by nifedipine. Alternatively, reduction in circulating insulin with nifedipine may be simply due to an improvement in

insulin sensitivity in these mice, as manifested by the fall in the plasma insulin to glucose ratio (Table 5). Similarly, clinical studies have demonstrated that Ca^{2+} channel blockers, including nitrendipine and diltiazem, induce a reduction of fasting serum insulin levels without changing fasting serum glucose and improve insulin sensitivity in obese hypertensive men and women (34, 35). Furthermore, an anti-obesity effect of Ca^{2+} channel antagonists has been reported in rodents (36, 37), even though the mechanism was not clear. Therefore, it is difficult to distinguish the nifedipine effect on the primary genetic lesion from secondary phenotypic abnormalities, such as hyperinsulinemia in the transgenic mice.

We have recently demonstrated that agouti stimulates FAS activity in a $[Ca^{2+}]i$-dependent fashion (13). Accordingly, it may be inferred that our observations of reduced FAS mRNA levels and activity and reduced adiposity in the transgenic mice on nifedipine diet result from inhibition of agouti stimulation of FAS expression. However, since the nifedipine treatment also normalized the hyperinsulinemia found in the transgenic mice, the reduction in circulating insulin is likely to have also contributed to the decrease in FAS activity and body fat. Indeed, it is likely that this anti-obesity effect of nifedipine may have resulted both from inhibition of agouti-induced Ca^{2+} influx in adipocytes and from the hypoinsulinemic effect, as we have found agouti and insulin to exhibit synergistic effects in stimulating FAS gene transcription (unpublished observation).

Several studies have identified Ca^{2+} regulatory elements that may mediate either negative or positive regulation of gene transcription by Ca^{2+} (38, 39). Therefore similar mechanisms may be involved in regulation of FAS gene transcription by Ca^{2+} and agouti.

Alternatively, it is also possible Ca^{2+} and agouti may affect FAS transcription in an indirect way, perhaps, though stimulation or inhibition of other genes in their target tissues. For example, an accumulation of $[Ca^{2+}]i$ inhibits the lipoprotein lipase expression in adipocytes (40), thereby decreasing fatty acid entry from triglyceride-rich-lipoprotein. This may also result in FAS suppression, as fatty acids are potent inhibitors of FAS gene expression (41-43).

In conclusion, data from the present study demonstrate that antagonism of Ca^{2+} influx may prevent agouti-mediated *de novo* lipogenesis and insulin resistance and thereby attenuate the increase in adiposity associated with dominant agouti mutations.

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PART 4

CONCLUSIONS

CONCLUSIONS

Obesity is strongly associated with the development of insulin resistance and type II diabetes, and a causal relationship between obesity and the subsequent development of glucose intolerance and insulin resistance is generally accepted (see literature review for references). Moreover, both obesity and insulin resistance are significant risk factors for the development of hypertension and coronary heart disease. This obesity syndrome imposes an enormous burden on the health care system. However, its extremely complex pathophysiologic nature and variable genetic mode of the penetrance of this disease has resulted in slow progress regarding the understanding mechanism. However, studies presented here support the hypothesis that altered $[Ca^{2+}]i$ metabolism is related to the pathogenesis of obesity and insulin resistance, thereby providing a potential target for the subsequent development of intervention.

Laboratory animals, especially single-gene mutations, have played an important role for understanding the pathophysiology of multifactorial human disease. In the field of obesity research, the cloning of the agouti gene provided an exciting opportunity not only for elucidation of the mechanism of obesity characteristic of dominant agouti mutation, but also for understanding the control of normal cellular energy metabolism. Based on the data from the present study, Ca^{2+} appears to play an important role in energy metabolism and the ability of the agouti protein to influence $[Ca^{2+}]i$ levels or activate Ca^{2+} channels appears to be responsible for efficient conversion of metabolic energy into fat in viable yellow mice. This agouti-mediated acceleration of fat depot storage appears to be, at least partially, via Ca^{2+} activation of fatty acid synthase (FAS) transcription.

Further studies of the precise role of Ca^{2+} in regulating FAS will contribute to understand the mechanism by which agouti protein stimulates adipocyte fat accumulation. The anti-adiposity effect of Ca^{2+} channel inhibitor in agouti-induced obesity observed in this study will be further clarified when tissue specific Ca^{2+} channel blockers are developed in order to distinguish between the potential roles of agouti-induced insulin secretion versus direct agouti-induced lipogenesis. The only well characterized function of agouti, regulation of mouse coat color, is based upon competitive antagonism of α-melanocyte stimulating hormone (α-MSH) binding. However, data from the present study indicate that agouti modulation of $[Ca^{2+}]i$ signaling is not based upon such an antagonism, but may be dependent upon a non-competitive interaction with one or more melanocortin receptor(s). This proposed relationship is summarized in Fig. 14. Nonetheless, in vivo studies of the effects of α-MSH and other melanocortins in animals exhibiting the obesity phenotype characteristic of dominant agouti mutations are still necessary to determine the potential role of melanocortin antagonism in agouti-induced obesity.

The present study may be viewed one step toward bridging the gap between molecular biology and genetics and physiology. Virtually, all of the currently known single genes causing obesity in mice, including agouti (A^{y}, A^{iapy}, A^{iy}, A^{sy}, and A^{vy}), obese (*ob*), diabetes (*db*), fat (*fat*), and tubby (*tub*), have recently been cloned, providing a new opportunity to couple genetic and functional analysis to physiology of tools in the field of obesity research. Using this with each of those mutation is anticipated to provide multiple therapeutic targets and opportunity for intervention in obesity.

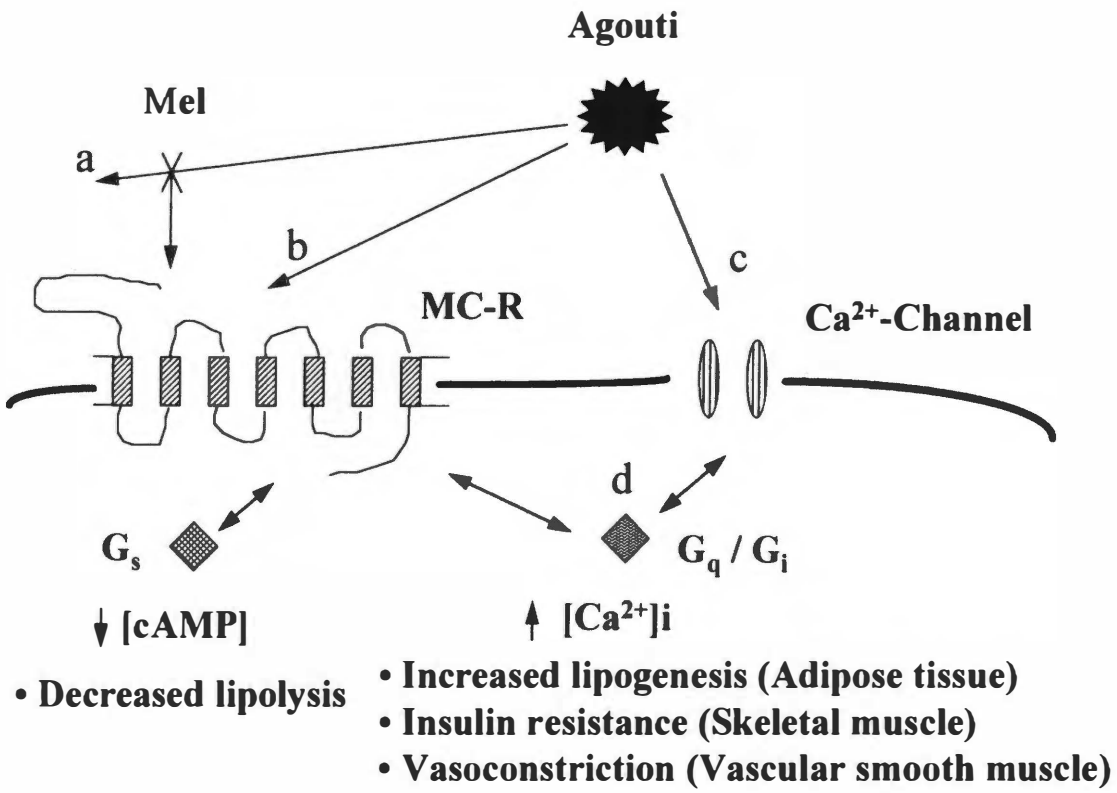

Figure 14. Proposed model for agouti action. Agouti may (a) inhibit melanocortin receptor binding or exert direct effects on (b) melanocortin receptors of (c) Ca^{2+} channel(s). There is proposed G-protein linkage (d) between a melanocortin receptor and a Ca^{2+} channel, such that binding of a agouti modulates the activity of the partnered receptor.

VITA

Jung Han Kim was born October 2, 1962 in Seoul, Korea the first of three children. She attended Chung-Ang Elementary School, Eue-Jung-Boo, Kyung-Ki-Do and Hwa-Ke Elementary School, Seoul. She graduated from Dobong Women's Middle School, Seoul and Hansung Women's High School, Seoul in February, in 1978 and 1981, respectively. In March of 1981, she attended Dongduck Women's University, Seoul, where she earned a bachelors degree and a master degree in Food and Nutrition Science in February of 1985 and 1987, respectively. She began her doctoral work in August, 1991 and defended her dissertation in May, 1996.