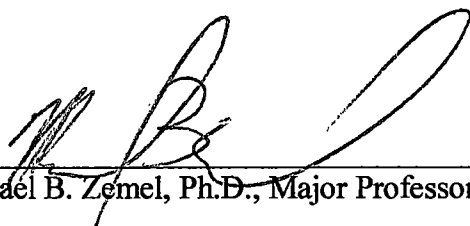
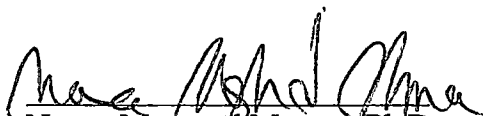
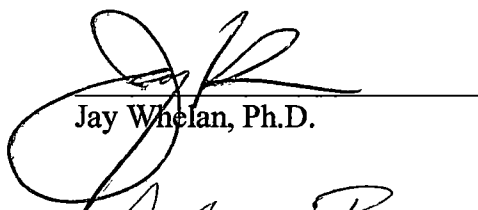
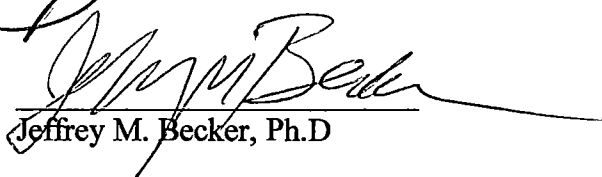


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AGOUTI MODULATION OF BOTH ADIPOCYTE AND PANCREATIC ISLET
FUNCTION VIA A Ca^{2+} -DEPENDENT MECHANISM

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

Bingzhong Xue
August 2000

DEDICATION

This dissertation is dedicated to my husband Hang Shi, my mother Guizhen Ju, and to the
memory of my father, Gongxi Xue

It is also dedicated to my father-in-law Guangrong Shi and my mother-in-law Yulan Gu

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ABSTRACT

Dominant mutations at the promoter of the mouse agouti gene results in a yellow coat color as well as obesity, hyperinsulinemia, insulin resistance and gender-dependent hyperglycemia. Both central and peripheral actions of agouti contribute to agouti-induced obesity syndrome. The human analogue of agouti is expressed in adipose tissue and exerts potent peripheral effects. Recombinant agouti protein has been shown to stimulate adipocyte intracellular calcium ($[Ca^{2+}]_i$) and up-regulate fatty acid synthase (FAS), a key enzyme in *de novo* lipogenesis in human adipocytes via a Ca^{2+} -dependent mechanism, thereby promoting lipid storage in adipose tissue. However, transgenic mice overexpressing agouti only in adipose tissue under the control of aP2 promoter do not become obese unless hyperinsulinemia is concomitantly present, suggesting an interaction between hyperinsulinemia and adipose tissue agouti expression. In addition, adipocyte lipolysis is also impaired in agouti mutants compared to wild type animals. We therefore further investigated the effect of recombinant agouti protein on both human adipocyte and pancreatic islet function. We demonstrated that the human homologue of agouti is expressed in the pancreas. Agouti protein also induced a significant Ca^{2+} influx into human pancreatic islets, and serves as a potent secretagogue for both insulin and amylin release in human pancreatic islets, which may contribute to hyperinsulinemia and hyperamylinemia. In human adipocyte, recombinant agouti protein potently inhibited both ACTH- and forskolin-induced lipolysis via a Ca^{2+} -dependent mechanism, indicating a post receptor event. Further investigation of the mechanism whereby increasing in $[Ca^{2+}]_i$ results in inhibition of lipolysis demonstrated that the anti-lipolytic effect of increasing $[Ca^{2+}]_i$ is exerted by the activation of adipocyte phosphodiesterase (PDE 3B),

leading to a decrease in cAMP, a net decrease in hormone sensitive lipase phosphorylation and inhibition of lipolysis. In addition, we demonstrated that human adipocyte agouti is up-regulated during adipocyte differentiation. Using human adipose tissue obtained from plastic surgery with body mass index (BMI) ranging from 21 to 31, we found a positive correlation between adipose tissue agouti content and FAS activity, as well as between adipose tissue agouti mRNA level and FAS mRNA level. These data suggest that agouti may modulate both adipocyte and pancreatic islet function via a Ca^{2+} -dependent mechanism. In the pancreas, agouti-induced hyperinsulinemia and hyperamylinemia may serve as a compensatory system in regulating blood glucose by coordinately regulating glucose input and clearance, respectively. However, agouti-induced hyperinsulinemia may contribute to insulin resistance and obesity. In addition, agouti stimulation of amylin release may result in islet amyloid deposits, impaired β -cell function and diabetes. In the adipose tissue, agouti potently inhibits agonists-stimulated lipolysis by activating adipocyte PDE 3B, thereby resulting in a coordinated regulation of adipocyte lipid metabolism, which may contribute to adipose tissue lipid storage. In addition, we have shown a correlation between human adipose tissue agouti expression and FAS activity and expression. As humans normally express agouti in adipose tissue and pancreas, it is possible that agouti and FAS may be regulated in a similar manner. Alternatively, agouti may be one of adipocyte synthesized factors that may in part contribute to the modulation of adipose tissue lipid metabolism in vivo. Finally, agouti-induced hyperinsulinemia may synergistically act with adipocyte agouti in modulating adipocyte lipid metabolism, thereby contributing to obesity.

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PART 1

INTRODUCTION

INTRODUCTION

The mouse *agouti* gene encodes a 131 amino acid secreted protein that is transiently expressed in neonatal skin and acts as a paracrine factor to regulate pigment production in melanocytes (1). However, dominant mutations at the promoter region of this gene results in ectopic expression of normal *agouti* transcripts in animal models such as the *lethal yellow* (A^y) and *viable yellow* (A^{vy}). This results in a yellow coat color and metabolic derangements including obesity, hyperinsulinemia, insulin resistance and gender-dependent hyperglycemia (2,3). In addition, transgenic mice overexpressing *agouti* under the control of β -actin promoter recapitulated the yellow coat as well as the obesity/diabetes syndrome, indicating that overexpression of *agouti per se* is responsible for this yellow obese syndrome (4).

Both central and peripheral actions of *agouti* contribute to *agouti*-induced obesity syndrome. Recent evidence indicates that chronic antagonism of central melanocortin 4-receptor (Mc4-r) may be responsible for the hyperphagia and obesity in yellow *agouti* mutants (5,6). However, recent data showed that Mc4-r is expressed in several peripheral tissues, including adipose tissue (7). It is therefore possible that *agouti*-induced obesity syndrome may also involve its action at peripheral melanocortin receptors. In addition, recent evidence also shows that *agouti* regulation of several peripheral cellular functions requires functional melanocortin receptors, but appears to be independent of melanocortin receptor antagonism (9).

The human homologue of *agouti* is expressed in adipose tissue (8). Our laboratory has previously shown that recombinant *agouti* protein increases intracellular calcium ($[Ca^{2+}]_i$) in several cell types (9), including murine and human adipocytes. *Agouti* protein

also stimulated lipogenesis in both murine and human adipocytes via a Ca^{2+} -dependent mechanism (10), thereby promoting lipid storage in adipose tissue.

However, transgenic mice expressing agouti in adipose tissue under the control of the aP2 promoter do not become obese unless hyperinsulinemia is concomitantly present by either daily insulin injection (11) or high sucrose feeding (12), indicating an interaction between hyperinsulinemia and adipocyte agouti expression in agouti-induced obesity syndrome. In agreement with this, *in vitro* studies also demonstrate additivity of agouti and insulin effects on lipogenesis (13). Hyperplasia of pancreatic β -cells precedes the development of obesity in agouti mutant mice (14). In addition, increasing $[\text{Ca}^{2+}]_i$ is the proximate signal for insulin release (15). Consequently, it is possible that agouti may exert direct effect on pancreatic islets to modulate Ca^{2+} -signaling and islet function.

Hyperglycemia does not occur until β -cell function is also impaired. Islet amyloid polypeptide, also called amylin, is the major component of islet amyloid deposits, which accumulates extracellularly between β -cells and capillaries in the islets of most type II diabetic patients (16). It is co-localized with insulin in β -cell secretory granules (17) and co-secreted with insulin in response to glucose and non-glucose secretagogues (18). Plasma amylin levels are elevated in agouti mutants (19). In addition, overexpression of human amylin in the pancreas of agouti mutants results in accelerated islet amyloid deposition, severely impaired β -cell function and diabetic phenotype (20). Further, since amylin release is regulated similarly to that of insulin, it is possible that agouti may also modulate pancreatic islet function by modulating amylin release.

Earlier studies showed that both basal and agonist-stimulated adipocyte lipolysis is impaired in agouti mutant mice compared to their wild-type littermates (21-23). Adipose

tissue from obese A^{vy} animals exhibit lower lipolytic rate in response to β -adrenergic receptor agonist epinephrine and phosphodiesterase inhibitor theophylline. However, their response to dibutyryl cAMP, a non-hydrolyzable cAMP analogue is normal, suggesting that obese animals have a defect in either the synthesis or the maintenance of cAMP (22). In addition, several reports demonstrated that increasing in $[Ca^{2+}]_i$ inhibited adipocyte lipolysis (24-27). It is possible that agouti may modulate adipocyte lipolysis, leading to a coordinated control of adipocyte lipogenesis and lipolysis.

Consequently, these studies investigated: (1) whether agouti is expressed in human pancreas; (2) the role of agouti protein in modulating Ca^{2+} -signaling and insulin and amylin release in human pancreatic islets; (3) whether agouti inhibits lipolysis in human adipocytes; (4) mechanisms of agouti and $[Ca^{2+}]_i$ modulating adipocyte lipolysis; (5) the relationship between human adipocyte differentiation and adipocyte agouti content and (6) the relationship between agouti expression and fatty acid synthase expression and activity human adipose tissue.

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PART 2
LITERATURE REVIEW

I. PHYSIOLOGICAL CHARACTERIZATION OF THE YELLOW OBESE MOUSE

The murine *agouti* locus in chromosome 2 normally controls the relative amount and distribution of the melanin pigment, eumelanin (black) and phaeomelanin (yellow), during hair growth (1). In wild-type mice, melanocytes located in hair follicles produce eumelanin at the beginning of hair growth. They subsequently produce phaeomelanin due to a transient expression of agouti during the mid-portion of the hair growth cycle. The melanocytes then switch back to eumelanin production, resulting in a typical agouti pigmentation of black hair with a subapical yellow band (2). The *agouti* gene encodes a novel paracrine signaling molecule which is produced by follicular non-pigment cells and act at melanocytes from within the follicular environment (3). At least 34 different dominant and recessive *agouti* locus alleles have been identified (4), resulting in a phenotypic dominance hierarchy in which phaeomelanin synthesis is generally dominant over eumelanin synthesis.

The most dominant mutations are *lethal yellow* (A^y) and *viable yellow* (A^{vy}). These mutations, along with several other mutations, *sienna yellow* (A^{sy}), *intermediate yellow* (A^{iy}), *hypervariable yellow* (A^{hvy}), *intracisternal A particle yellow* (A^{iapy}), are promoter mutations, leading to ectopic expression of a normal agouti protein and dominant inheritance of a yellow coat. These mutations result in a uniformly yellow fur in A^y , and variable coat colors in the other models ranging from completely yellow to various degree of intermixtures of yellow and black to a coat color referred to as “pseudoagouti” that closely resembles wild-type agouti (5-10). In addition, mice carrying these dominant mutations develop a pleiotropic syndrome characterized by obesity, hyperinsulinemia, insulin resistance and diabetes (5).

A. OBESITY

1. Body weight and body triacylglycerol content

Mice with dominant agouti mutations start to show an increase in body weight at about 4 weeks of age, and peak between 8 and 17 months of age (11,12). Their body triacylglycerol content increases significantly, reaching about 26 % of the body weight in adult yellow mice, representing a 4.4-fold increase compared to wild type animals (13). The increase in adipose tissue is primarily due to hypertrophy, as obese yellow A^y mice have larger adipocytes than normal mice while the adipocyte number is not significantly different from that of normal mice (14). The enlargement of adipocytes is due to host environmental factors, rather than factors associated with the fat cell itself, as adipose tissue transplanted from yellow obese ($A^y/+$) mice to lean animals and vice versa acquires morphological characteristic similar to that of host (15).

In addition to marked elevations in fat content, yellow mice have increased linear growth (16), muscle mass (17) and fat-free dry weight (18,19). These observations indicate that dominant mutations at the agouti locus results in alterations of their metabolic dynamics which favors anabolic reactions (20).

2. Hyperphagia and energy efficiency

Yellow obese mice are moderately hyperphagic, eating about 30 % more than their wild type littermates (21). However, this is not sufficient to account for the 4-fold increase in body triacylglycerol content in these mice, indicating an increased efficiency in storing energy into body fat. Indeed, yellow obese mice exhibit an approximately 4-fold increase in the efficiency of converting food energy to body weight compared to wild type animals (21).

Feeding yellow A^y mice either a high sucrose or a high fat diet results in more weight gain than A^y mice on a standard diet due to more increased energy efficiency (21). In addition, Carpenter et al reported that yellow mice gain more weight on a high-fat diet than on high-carbohydrate or high-protein diets (16), indicating that they are sensitive to dietary fat content. Further, when given three-choice macronutrient diet of pure fat, carbohydrate, and protein, A^y/a mice showed an increased preference of fat intake, in that they gained more weight and consumed a greater proportion of their daily intake from fat and less from carbohydrate than wild-type littermates (22), although the mechanism of this preferential nutrient partitioning is not clear.

3. Lipogenesis, lipolysis and lipid metabolism

The expression level of fatty acid synthase (FAS) and stearyl-CoA desaturase (SCD), two key enzymes in fatty acid biosynthesis and desaturation, respectively, are elevated in liver and adipose tissue of obese A^y mice (23). Increases in liver weight and elevations in liver lipogenic enzyme activities, such as 6-phosphogluconate dehydrogenase, fatty acid synthetase, citrate cleavage enzyme, glucose-6-phosphate dehydrogenase, acetyl CoA carboxylase and malic enzyme, has been reported by others (24). These data suggest an increase in lipogenesis rate in both liver and adipose tissue in these mice.

However, unlike *ob/ob* and *db/db* mice, which are characterized by juvenile-onset of obesity, the yellow mice exhibit maturity-onset obesity (13). *Ob/ob* and *db/db* mice have higher lipogenesis rates and accumulate more triacylglycerol when they are young, leading to a more rapid weight gain; their body triacylglycerol content reaches 50 % of their body weight. At maturity, their lipogenesis rates decrease to normal. In contrast,

young yellow mice do not have higher lipogenesis rates and only have a moderate amount of triacylglycerol stored. They do not gain weight rapidly as they mature. However, both carcass and hepatic lipogenesis rate in yellow mice remains constant; since lipogenesis decreases with maturation in normal mice, this results in increased lipogenesis in mature yellow mice compared to control (13).

Adipose tissue from yellow A^y mice exhibits impaired basal lipolytic rate compared to that of a/a mice (25). The response of A^y adipose tissue to epinephrine (25), theophylline (25), and the β -agonist LY79771 (26) and LY104119 (27) is lower than normal. However, the response to dibutyryl cyclic AMP is normal (25). These observations indicate that adipocytes in yellow A^y mice may have a defect in the generation and/or maintenance of cyclic AMP. Consistent with this, adipose tissue from yellow A^y mice have significantly lower cyclic AMP level when stimulated with LY79771 (26) and LY104119 (27).

In addition to increased lipogenesis and decreased lipolysis, obese yellow mice also have elevated fed and fasting blood total lipid level, and female yellow mice exhibit hypercholesterolemia (16). Obese yellow mice also have non-fasting hyperketonemia (16).

4. Thermogenesis

One study showed that yellow obese mice exhibit a defect in adaptive thermogenesis, as their rectal temperature continues to fall during cold exposure while that of normal mice only falls temporarily (28). However, others did not find any difference in thermogenic enzyme activity in brown adipose tissue between yellow obese (A^y) mice and wild type animals (29). In addition, the thermogenic response to an β -

adrenergic receptor agonist, LY104119, is not different between yellow obese (A^y) mice and wild type mice (27). Further, energy restriction in yellow A^y mice results in weight changes that is not paralleled with changes in thermogenesis (27). These observations suggest that decrease in thermogenesis may not be sufficient to account for the weight gain in yellow obese mice; instead, enhanced metabolic efficiency in converting food energy to body fat appears to play a major role in these yellow obese mice.

5. Endocrine status

Studies by Wolff et al demonstrated that parabiosis between yellow and non-yellow littermates for approximately six months failed to affect body weight and body composition in either group (30). This observation indicates that circulating hormones are not directly involved in the development of obesity; in addition, it confirms that a functional agouti protein is not shared in the circulation between the parabiotic partners, consistent with the paracrine nature of agouti in regulating pigmentation.

Hypophysectomy at 45-50 days of age results in significant decrease of body weight in obese yellow (A^y/a) mice (31). However, hypophysectomized yellow mice still gain more weight than hypophysectomized nonyellow siblings and show excessive accumulation of fat, demonstrating that the pituitary mediated endocrine pathways are not primarily involved in the development of yellow obesity (31). Similarly, Salem et al. found that hypophysectomy in yellow A^y mice results in significant weight loss; however, their parametrial fat pad is still heavier than that of wild type animals (32).

Plasma corticosterone level in A^y mice is moderately elevated (33). In addition, adrenalectomy in yellow A^y mice resulted in a 33 % reduction of body fat; however, adrenalectomized yellow mice were still fatter and had heavier fat pad mass than

adrenalectomized wild type mice (33), suggesting a secondary role for the adrenal gland in the development of obesity in yellow mice.

Growth hormone and thyrotropin do not appear to be involved in the development of obesity in yellow mice. Yellow hypophysectomized mice still have increased linear growth and fat accumulation (31,32). In addition, yellow dwarf (A^y/a dw/dw) mice, which have both the yellow obese mice gene (A^y) and congenic growth hormone deficiency, become obese despite their stunted growth (34).

Plasma insulin level started to increase in obese yellow (A^y) mice at 6 week of age (35), while hyperinsulinemia appears in db/db mice at 10 days of age (36) and in ob/ob mice at 4 weeks of age (37). At 6 month of age when obesity in yellow mice peaks, plasma insulin level is elevated more than 20-fold compared to controls (38). As insulin promotes nutrient partitioning into adipose tissue and stimulates adipocyte growth and development (39), the hyperinsulinemia in the yellow agouti mice may contribute to their obesity. Indeed, Yen et al. found a positive correlation between plasma insulin level and hepatic lipogenic enzyme activity and between plasma insulin level and body weight (24). In addition, pancreatic β -cell hyperplasia appears between 14 and 21 days of age in yellow A^y mice, before any detectable weight gain or changes in insulin or glucagon levels (40). However, the relationships between hyperinsulinemia, insulin resistance and obesity are complex, and the role of insulin in obesity in yellow obese mice is not clear.

Leptin is produced primarily by adipose tissue (41) and plays a key role in regulation of food intake and energy balance. In yellow A^y/a mice as well as other obese animal models such as db/db , fa/fa , ventrimedial hypothalamus (VMH)-lesioned, those induced by diet, gold thioglucose, monosodium glutamate, transgenic ablation of brown

adipose tissue and in obese humans, leptin expression and circulating level are increased (42). This increase in leptin correlates well with adiposity (42). In addition, yellow A^y/a mice fail to respond to subcutaneous leptin injection and are only 1 % as sensitive to intracerebroventricular leptin infusion compared to the decrease in food intake and weight loss observed with leptin administration in wild type animals (43). These observations suggest a leptin resistance in yellow obese mice. However, leptin-resistance appears to result from receptor desensitization secondary to increased circulating leptin, not an intrinsic defect in leptin signaling, as removal of leptin from the A^y/a mice in the double mutant *lethal yellow/leptin deficient* model ($A^y/a \text{ } lep^{ob}/lep^{ob}$) completely restored the responsiveness of these animals to exogenous leptin (44).

B. Diabetes

1. Hyperinsulinemia and hyperamylinemia

Yellow obese mice exhibit significant hyperinsulinemia as described above. In addition, islet amyloid polypeptide, also called amylin, is another pancreatic β -cell hormone that is co-localized with insulin in β -cell secretory granules (45) and co-secreted with insulin in response to glucose and non-glucose secretagogues (46). Recent data have shown that amylin coordinates with insulin in regulating blood glucose by slowing the rate of gastric emptying (47) and inhibiting glucagon release (48), resulting in reduced postprandial glucose level. However, little data are available regarding amylin status in yellow obese mice. Gill et al reported that plasma amylin levels in yellow A^y mice at 6 ½ month of age are 4-5 fold that of normal mice (38), resulting in an increase in the insulin/amylin ratio (38). In another study, Soeller et al reported that plasma amylin level in A^y mice at 1 year of age is about 3-fold that of normal mice (49).

2. Insulin resistance and hyperglycemia

Fasting blood glucose levels are normal in yellow obese mice; however, fed blood glucose levels are usually elevated in these mice (16,35). Yellow obese mice exhibit a sexual dimorphism in blood glucose level. While male mice are usually hyperglycemic, female mice are either normal (35) or only mildly hyperglycemic (38). In addition, glycemic response to growth hormone, ACTH, cortisone and glucagon is exaggerated in yellow obese mice, with yellow males showing a greater hyperglycemic response (35). Further, during glucose tolerance test (GTT), yellow male A^{vy} mice at 12 week of age showed a greater impairment in glucose tolerance than female mice (35). This can be ascribed to the higher blood insulin levels in female mice at that age (35). However, the difference in blood glucose level could not be solely attributed to insulin levels. As some studies found more prominent hyperinsulinemia in female yellow mice (38), others reported that male yellow mice have higher plasma insulin levels than female mice at 5-9 weeks of age and exhibit more profound hyperglycemia than females at the same age (35). Indeed, administration of dexamethasone induces hyperglycemia in female yellow mice (50), which is paralleled with the induction of hepatic estrogen sulfotransferase, an enzyme that inactivates estrogen (51), suggesting a protective role for estrogen against the development of hyperglycemia in female yellow mice.

Glucose tolerance in yellow mice of various ages (3-8 months) remained impaired despite the presence of hyperinsulinemia (400-500 μ U/ml), indicating the existence of an insulin resistant condition (35). In addition, adipose tissue from 4-month old yellow obese mice require significantly higher insulin concentration to stimulate glucose oxidation (35), confirming yellow obese mice is less sensitive to insulin.

C. Hypertension

Obesity-induced hypertension is commonly attributed to insulin resistance and hyperinsulinemia (52). However, fewer data is available regarding blood pressure in obese animal models including yellow obese (A^y) mice. Mark et al studied body weight and arterial pressure in age-matched (14 weeks) *ob/ob* mice and A^y/a mice with the same background (C57BL/6J) (53). Leptin-deficient *ob/ob* mice have lower arterial blood pressure than their wild type littermates although they are profoundly obese. In contrast, yellow mice have significantly higher arterial pressure and heart rate than their lean controls despite the fact that their obesity is milder than *ob/ob* mice (53). This observation suggests that obesity does not invariably increase arterial pressure in mice; instead, the arterial pressure response to obesity may depend on the underlying genetic and neuroendocrine defects (53). Indeed, *in vitro* data demonstrate that leptin possesses pressor actions (54) and increase arterial pressure when infused chronically (55,56) or over-expressed transgenically (57). In addition, agouti protein has been shown to potentiate the vasoconstrictor action of pressor agonists in vitro (58). These observations may explain the difference in arterial pressure in the two obese animal models.

II. THE MOUSE AGOUTI GENE AND ROLE OF AGOUTI IN OBESITY

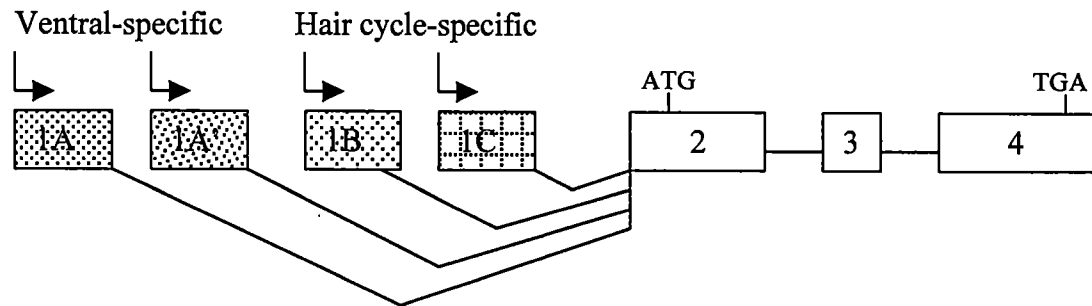
A. Molecular characteristic of the agouti gene and agouti dominant mutations

1. The mouse agouti gene and its product

Agouti was the first obesity gene to be cloned (59,60). The gene has three coding exons 2, 3 and 4 (Figure 1A). The utilization of different promoters and alternative splicing of the first non-coding exon gives rise to ventral-specific and hair-cycle-specific agouti transcripts that range in size from 0.7 to 0.8 kb with identical coding sequences (Figure 1A) (61,62). There are two wild-type alleles in the agouti locus, *agouti* (*A*) and *light-bellied agouti* (*A^w*). In mice with *agouti* genotype (*A*), yellow pigment is synthesized only during the midportion of hair growth cycle due to the expression of hair-cycle-specific transcript, resulting in agouti hair in both dorsal and ventral regions. In contrast, the ventral surface of mice carrying *light-bellied agouti* (*A^w*) alleles has entirely yellow hairs due to the expression of ventral-specific agouti transcript while their dorsal hairs are agouti due to the expression of hair-cycle-specific agouti transcript (61,62).

The murine agouti gene is located on chromosome 2 and encodes a 131 amino acid polypeptide (Figure 1B) (59,60). The protein contains a 22-amino acid signal peptide, characteristic of secreted proteins, a highly basic central region, a poly-proline rich stretch that follows the basic region and a cystein-rich carboxyl-terminal region. The Asn 39 is a putative N-linked glycosylation site, consistent with the purified protein having a larger apparent mass (18.5 kDa) than predicted (11.8 kDa) (60,63). All 10 cysteines at the carboxyl terminal region are involved in forming disulfide bonds (63). The spacing between cystein residues and the pattern of disulfide bond resemble that of neurotoxins

A



B

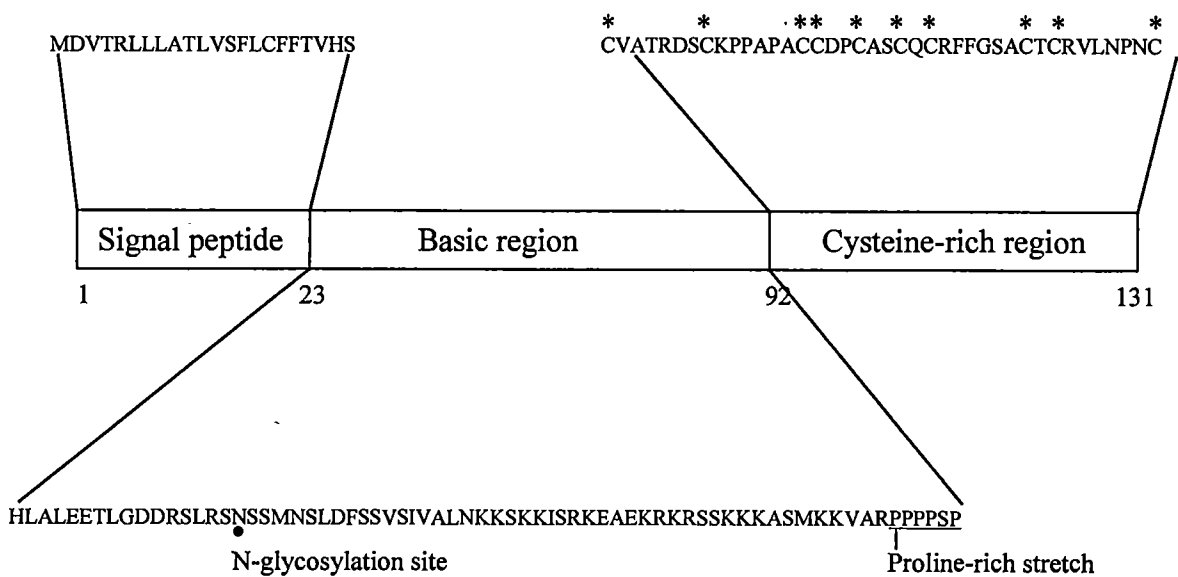


Figure 1. Genomic and protein structure of agouti. A. Genomic structure of the mouse agouti gene. Alternative splicing of the first non-coding exon gives rise to ventral-specific (1A and 1A') and hair cycle-specific (1B and 1C) transcripts. The start and stop codons have been indicated in exons 2 and 4. B. Agouti protein structure. • putative N-glycosylation site. * Cysteine residues. Underline: proline-rich stretch. 1A, 1A' and 1B, 1C: ventral-specific and hair cycle-specific non-coding exon 1, respectively. 2, 3, and 4: coding exons.

found in the venom of the primitive hunting spiders and cone snails; these neurotoxins (conotoxins and plectoxins) primarily target calcium channels (63,64).

2. Agouti dominant mutations and epigenetic regulation

In wild type mice, agouti is expressed in neonatal skin during hair growth (59,60). Agouti expression is also detected in adult testis (59, 60) and during embryonic development (65). The expression of agouti appears to be in hair follicles and the nonkeratinized epidermis. The expression in hair follicles appears to include the dermal papillae, the inner and outer root sheaths and the hair cortex, which is continuous with the epidermis and appears to include basal and suprabasal cells. There is no expression detectable in the extrafollicular dermis (60).

However, dominant mutations at the *agouti* locus, such as *lethal yellow* (A^Y), *viable yellow* (A^{vy}), *sienna yellow* (A^{sy}), *intermediate yellow* (A^{by}), *hypervariable yellow* (A^{hvy}) and *intracisternal A particle yellow* (A^{iapy}), which are regulatory mutations at the promoter region, result in ubiquitous expression of normal agouti transcript. This ectopic expression leads to a yellow coat color and metabolic derangements including obesity, hyperinsulinemia and insulin resistance (5-10).

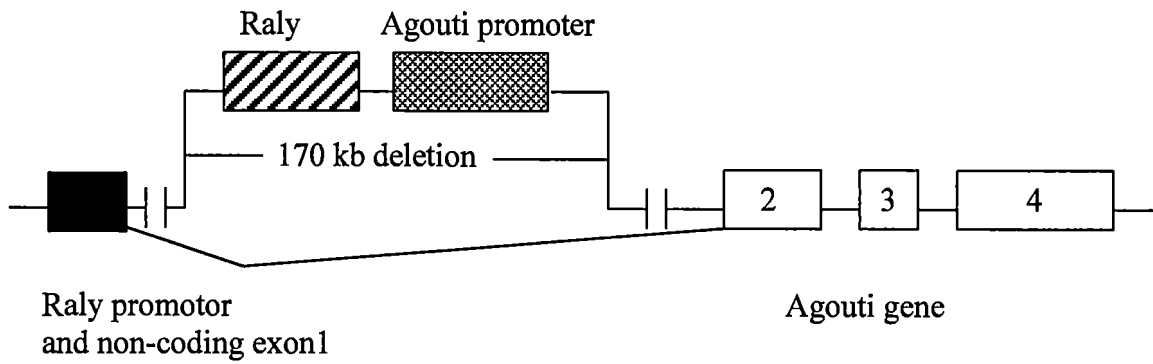
Lethal yellow (A^Y) homozygotes die around time of implantation (66,67). In multiple tissues of A^Y heterozygotes, a mRNA transcript of about 1.1 kb is detected in adult animals. The increase in size is due to the replacement of novel sequences at the 5' untranslated region of the A^Y agouti transcript, while the exons 2-4, which contain the 131-amino-acid open reading frame (ORF), is identical to the wild type agouti transcript (59,60). Additional experiments reveals that A^Y transcript-specific novel sequences at the 5' untranslated region corresponds to the upstream untranslated region of a gene called

Raly (*Ribonucleoprotein Associated with Lethal Yellow*) (68), a ubiquitously expressed heterogeneous nuclear ribonucleoprotein, which normally maps 280 kb proximal to *agouti* on mouse chromosome 2 (68, 69). This chimeric *Raly/agouti* transcript results from a 170-kb deletion that removes all but the first noncoding exon of *Raly* (Figure 2A) (69). This results in the transcription from the *Raly* promoter and splicing of the *Raly* upstream noncoding region to the *agouti* coding exons (69). The deletion of *Raly* is responsible for the recessive embryonic lethality of the A^y mutation. In addition, the deletion of *Raly* put the expression of *agouti* under the control of *Raly* promoter, which is responsible for the ectopic and ubiquitous expression of the *agouti* gene, leading to the pleiotropic phenotype exhibited by A^y mice (68).

Unlike A^y , *viable yellow* (A^{vy}) homozygotes are viable. Analysis of the expression pattern reveals that A^{vy} mice express an *agouti* mRNA in all tissues examined that is indistinguishable in size from wild-type mice (10). Molecular cloning studies indicate that the A^{vy} -specific mRNA is chimeric, with a portion of the 5' non-coding sequence derived from an intracisternal A particle (IAP) that is inserted 100-kb upstream of *agouti* coding sequences (10). Further studies reveal that a 5.4-kb IAP is inserted into exon 1A with a transcriptional orientation opposite that of *agouti*, producing a chimeric *agouti* transcript in which the normal *agouti* splice site pattern is preserved (10) (Figure 2B). The promoter/enhancer within the long terminal repeat (LTR) of the IAP, which has bidirectional promoter activity, appears to be responsible for the ectopic expression of the normal *agouti* protein (10).

Molecular analysis of other *agouti* dominant mutations, such as A^{iy} , A^{hvy} and A^{iapv} demonstrates that they are also derived from the insertion of IAP elements upstream of

A



B

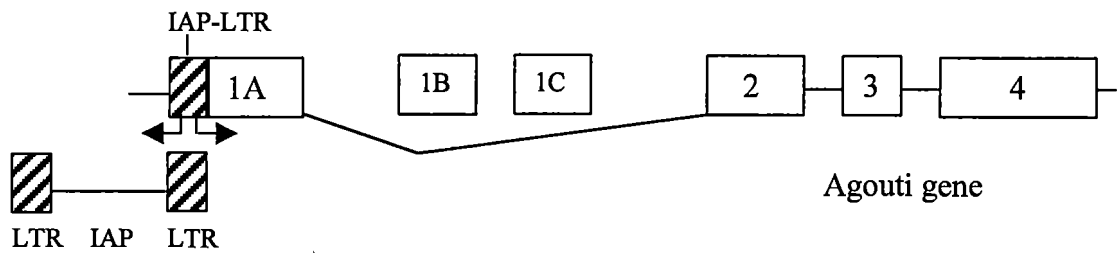


Figure 2. Proposed models for agouti dominant mutations. A. The A^y mutation results from the 170 kb deletion which removes agouti promoter and most of the Raly gene except the first non-coding exon. B. The A^w mutation results from the insertion of an IAP element in Exon 1A. IAP: intracisternal A particle. LTR: long terminal repeats. 1A, 1B and 1C: ventral- and hair cycle-specific non-coding exon 1. 2, 3, and 4: coding exons.

the coding region, which are responsible for the ectopic expression of a normal agouti protein (6, 9,10). In contrast, A^{vy} mutation is caused by an insertion of novel DNA sequences 10-15-kb upstream of agouti exon 2 that is not similar to any sequence in the public databases (10).

Dominant mutations caused by A^{vy} (5,10), A^{iy} (7), A^{hvy} (8,9) and A^{iapy} (6) exhibit a variability in coat color that ranges from totally yellow to various degrees of mottling to a coat referred to as "pseudoagouti" that is not distinguishable from wild-type agouti. The degree of expression of agouti is somewhat correlated with the expression of the mutant phenotype both in coat color variation and obesity syndrome (70). The level of expression associated with A^{iy} is less than 50 % of that associated with A^{vy} , which correlates with the relative degree of obesity caused by these mutations (7,10). In addition, Pseudoagouti A^{vy} , A^{iy} , A^{hvy} and A^{iapy} mice do not become obese, whereas yellow A^{vy} , A^{iy} , A^{hvy} and A^{iapy} mice do develop the disorder (7, 8,70).

Although the precise mechanism of the apparent somatic reversion to lean pseudoagouti phenotype is not clear, low levels of agouti expression have been detected in all tissues of pseudoagouti A^{vy} mice using RT-PCR, although the expression can not be detected by northern blot with total RNA (10). This indicates that epigenetic inactivation of regulatory sequences within the IAP LTR rather than excision of IAP is responsible for the somatic reversion in these animals (10). Differences in strain background and parental origin, and differences in the site of insertion have been proposed as mechanisms (10). Recent studies demonstrate that differences in the methylation status of the inserted IAP LTR appear to be correlated with the differential expression of agouti phenotype (9,70). In yellow A^{iapy}/a and A^{hvy}/a mice, the IAP LTR is hypomethylated, whereas in

pseudoagouti A^{iapy}/a mice and black A^{hvy}/a homologs, the LTR is methylated (9, 70). In addition, the expressivity of the agouti allele is also influenced by maternal genotype and epigenetic phenotype at the *agouti* locus (70- 72). Wolff et al. summarized that pseudoagouti dams produce a considerably higher proportion of pseudoagouti offsprings than do yellow phenotype dams (72).

3. Transgenic mouse studies

To confirm that ectopic expression of agouti is sufficient to induce this pleiotropic syndrome, transgenic mice that overexpress agouti under the control of either β -actin (BAP) or phosphoglycerate kinase (PGK) promoter were generated (73). Analysis of several lines of transgenic mice revealed that they express high levels of agouti in every tissue examined. In addition, these mice develop yellow fur and become obese as they age. Transgenic males became 30 –40 % heavier and females 60-70 % heavier than nontransgenic controls. Further, both male and female transgenic mice become hyperinsulinemic, whereas males develop overt hyperglycemia. The ratio of insulin to glucose in the transgenic mice is twice that of the nontransgenic controls, indicating a profound insulin resistance status in these mice. Moreover, plasma leptin level is 5-6-fold higher in transgenic mice than in nontransgenic controls (74). These findings confirm that the ectopic expression of agouti *per se* is responsible for this yellow obese syndrome associated with dominant agouti mutations.

Mice specifically overexpressing agouti in the skin under the control of keratin-14 (K14) keratinocyte specific promoter developed yellow fur; however, these mice did not become obese (75). This observation further confirms that agouti acts as a paracrine factor that acts within local environment instead of a circulating protein, and that the

pleiotropic phenotype in dominant agouti mutants results from ectopic expression of agouti in novel tissues other than skin.

4. Human homologue of the mouse agouti gene

The human homologue of agouti has been cloned and mapped to chromosome 20 (76,77). The open reading frame, which encodes a protein of 132 amino acids, is 85 % identical to the mouse agouti at the nucleotide sequence level, and 80 % identical to the mouse agouti at the amino acid level (76, 77). However, the human regions corresponding to the 5' and 3' untranslated portions of the mouse mRNA diverge considerably (76, 77). The human agouti protein also contains a signal sequence at the amino terminus, indicative of a secreted peptide. The highly basic domain and the cystein-rich domain near the carboxyl terminus are also conserved between the predicted mouse and human proteins (76, 77). The human agouti is expressed in adipose tissue, heart, ovary, testis, and to a lesser extent, in foreskin, kidney and liver (76,77). However, unlike mouse agouti, the expression of human agouti in foreskin is not correlated with pigmentation phenotype (77). Transgenic mice overexpressing the human agouti gene under the control of human β -actin promoter developed yellow coat color (77) and obesity, suggesting that the human agouti protein is fully functional. The presence of agouti in normal human tissues indicates a possible physiological function of agouti in humans (77).

B. Mechanisms of agouti signaling

1. Melanocortin receptors and their ligands

a. The melanocortin receptors

Melanocortin receptors (MCRs) belong to G protein coupled seven transmembrane receptor family. The five distinct MCRs being cloned are expressed in a wide variety of tissues (78,79) (Table 1). Mc1-r, whose ligand is α -melanocyte stimulating hormone (α -MSH), is primarily expressed in melanocytes and melanoma cells from a variety of vertebrate organisms, and involved in the regulation of pigment production (78,79). Recent studies utilizing in situ hybridization, immunohistochemistry and reverse transcription-polymerase chain reaction (RT-PCR) demonstrated that Mc1-r is widely expressed in human tissues, including periaqueductal grey matter in central nervous system, pituitary and a variety of peripheral tissues (80-82).

Mc2-r, the receptor for adrenocorticotrophic hormone (ACTH), is highly expressed in adrenal cortex (80,83) and adipocytes (84), consistent with a role for Mc2-r in mediating the effect of ACTH on steroid secretion in adrenal gland and lipolysis in adipocytes. Loss of Mc2-r function in humans prevents the adrenal cortex from responding to ACTH, leading to familial glucocorticoid deficiency (85), confirming the role of Mc2-r in regulating adrenal cortex function. Recently, the expression of Mc2-r was identified in the skin (86,87). Further, in addition to α -MSH, which is well known to be expressed in the skin, ACTH-(1-39) and its fragments have also been demonstrated to be expressed in the skin (88). These findings suggest novel roles for Mc2-r and corticotropins in the physiology of the skin.

Table 1. Melanocortin receptor subtypes

Subtypes	Tissue distribution	Agonist profile
Mc1-r	widely expressed	α -MSH = desacetyl α -MSH > β -MSH = diacetyl α -MSH = ACTH = γ -MSH
Mc2-r	adrenal gland, adipocytes, skin, placenta, heart, testis, pancreas	ACTH
Mc3-r	brain, placenta, gut, adipocytes, pancreas, heart, testis	γ -MSH = desacetyl α -MSH = β -MSH = α -MSH > diacetyl α -MSH > ACTH
Mc4-r	brain, heart, adipocytes, Skeletal muscle, liver	desacetyl α -MSH = β -MSH = α -MSH = ACTH > diacetyl α -MSH > γ -MSH
Mc5-r	widely expressed	desacetyl α -MSH = α -MSH = diacetyl α -MSH > β -MSH = ACTH > γ -MSH

Northern hybridization analysis has demonstrated Mc3-r expression in brain and placenta, while the expression has been also detected in human stomach, duodenum and rat pancreas by RT-PCR (89). In situ hybridization demonstrated that Mc3-r is highly expressed in regions of the hypothalamus and limbic system. A high density of receptor expression is detected in the dorsomedial part of the ventromedial nucleus of the hypothalamus and the posterior hypothalamus, including the arcuate nucleus (90). Mc3-r expression is also found in septum, hippocampus, thalamus and midbrain (90).

Northern hybridization analysis demonstrated that Mc4-r is highly expressed in brain (91). Detailed analysis using in situ hybridization showed that Mc4-r is much more widely expressed in the rat brain than Mc3-r, in virtually every brain region (92).

Mc5-r has a wide distribution of expression in both central nervous system and peripheral tissues, and is found in many human tissues (80). Mc5-r expression is found in skeletal muscle and several endocrine and exocrine glands, including lachrymal, prostate, seminal, pancreatic, preputial, Harderian and thyroid glands (93,94). In addition, Mc5-r-deficient mice show an essential total loss of [^{125} I] Nle⁴, D-phe⁷-MSH (NDP-MSH, a synthetic melanocortin agonist) binding sites in skeletal muscle, Harderian, lachrymal and preputial glands (93), and show a reduced secretion by sebaceous glands, leading to defects in thermoregulation and water repulsion (93). This suggests that Mc5-r represents the majority of melanocortin receptors in these tissues.

Studies in mouse adipose tissue and 3T3-L1 adipocytes using RT-PCR and Northern hybridization with poly A⁺ RNA showed that both Mc2-r and Mc5-r are highly expressed in these tissues (84). However, the expression of Mc1-r, Mc3-r and Mc4-r can not be detected by RT-PCR (84). Chhajlani et al also found that human adipose tissue only

express Mc1-r and Mc5-r by RT-PCR (80). However, recent RT-PCR studies by Chagnon et al. showed that all 5 melanocortin receptors are expressed in human adipose tissue (82). In addition, Mc1-r, Mc2-r and Mc5-r expression is also detected in human pancreas by RT-PCR (82), while Mc3-r expression is detected in rat pancreas (89).

The five melanocortin receptors are 39-61 % identical to one another on the amino acid level, with Mc3-r, Mc4-r and Mc5-r appear more related to one another (55-61 %) than to the Mc1-r and Mc2-r (43-46 %), and the Mc1-r and Mc2-r are the most distantly related (39 %) (79). Each of the melanocortin receptors couples to activation of adenylyl cyclase. Konda et al. demonstrated that Mc3-r is also coupled to G_q , leading to an increase in inositol 1, 3, 4-phosphate production (95).

b. Melanocortin peptides

Natural melanocortin peptides are derived from pro-opiomelanocortin (POMC) by proteolytic cleavage (Figure 3). POMC is produced in pituitary, brain and several peripheral tissues including skin, pancreas and testis (96). POMC in the pituitary is the precursor of circulating α -MSH, ACTH and β -endorphin. POMC is processed into ACTH₁₋₃₉ in the corticotrophic cells of the anterior lobe of the pituitary, while α -MSH is the major end product in pituitary intermediate lobe melanotrophs, derived from the cleavage of amino acids 1-13 from ACTH₁₋₃₉ (96). The majority of α -MSH produced in pituitary intermediate lobe melanotrophs is acetylated at the amino terminus (monoacetyl- and diacetyl- α -MSH), while desacetyl α -MSH is the predominant form produced in the brain (97,98), fetus (99,100) and human blood (101,102). In Brain, POMC is produced in the neurons of the hypothalamic arcuate nucleus and the nucleus of the solitary tract of the brain stem (103), and appears to be processed differently to α -, β -

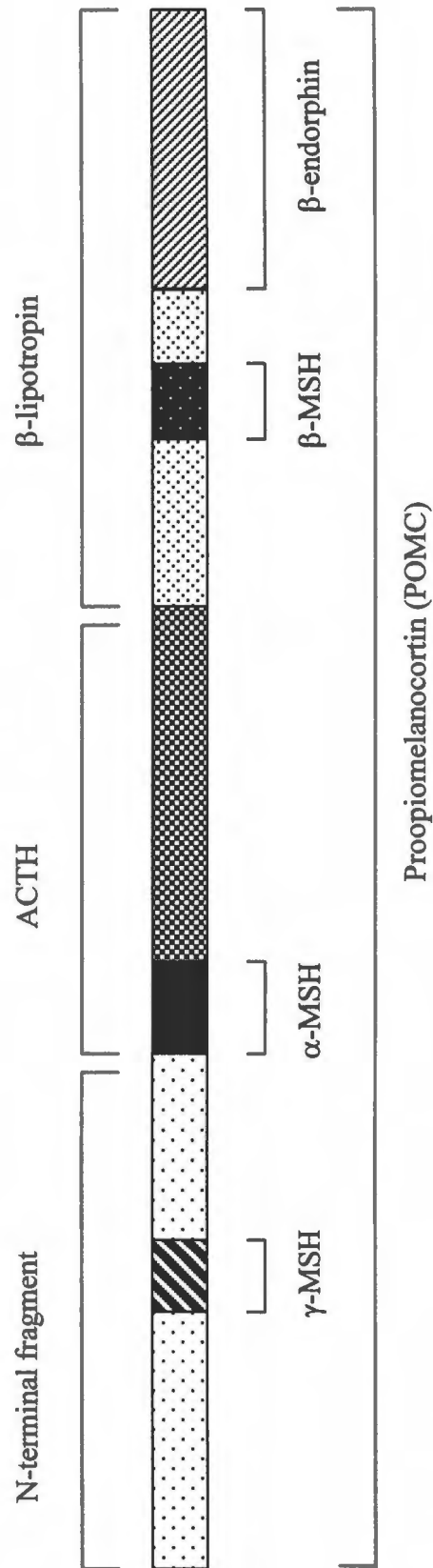


Figure 3. Melanocortin peptides and proopiomelanocortin (POMC).

and γ -MSH and ACTH in various brain regions (104-107). α -MSH immunoreactive perikarya are found in three regions: the hypothalamus arcuate nucleus, the dorsolateral region of the hypothalamus and the nucleus of solitary tract (105). β -MSH has been detected in human hypothalamus, and γ -MSH immunoreactive neurons are present at discrete locations in the central nervous system (105). In addition, the major form of α -MSH in mediobasal and dorsolateral hypothalamic region is desacetyl α -MSH, while amygdalanuclei, hippocampus, cortex and medulla oblongata regions primarily contain acetylated α -MSH (α -MSH + diacetyl α -MSH) (108).

N-terminal acetylation may alter the biological activity of peptide hormones and neuropeptides. Shimizu et al reported that food intake, weight gain and fat pad weight are increased by subcutaneous administration of desacetyl- α -MSH while α -MSH injections do not significantly increase food intake or body weight (109). In addition, administration of desacetyl α -MSH in adrenalectomized yellow A^{vy} mice restored epididymal and retroperitoneal fat pad mass to the levels similar to sham operated mice (33), while administration of α -MSH was without effect on fat pad mass in adrenalectomized A^{vy} mice (33). Further, Intracerebroventricular (ICV) administration of α -MSH and $^{1-24}$ ACTH inhibited food intake in food deprived (110) and fed (111) rats, whereas ICV injections of desacetyl- α -MSH had no effect. Bray et al found that the pituitary of yellow A^{vy} mice had a reduced α -MSH/desacetyl- α -MSH ratio (112) and hypothesized that interference in the acetylation of MSH would result in an elevation of desacetyl α -MSH, leading to hyperphagia and faster weight gain in yellow obese mice. However, Monroe et al found no evidence of an imbalance of des-, mono- and diacetylated α -MSH in the

pituitary of obese *A^y* mice (113). The physiological functions of different forms of MSH and their interaction with melanocortin receptors remain to be determined.

α , β , γ -MSH peptides are processed from different regions of the POMC precursor (Figure 3) to yield peptides sharing a conserved sequence, His-Phe-Arg-Trp (H-F-R-W), which is critical for melanocortin receptor binding and activation (78). The natural melanocortin peptides all bind the melanocortin receptors with an order of potency $Mc1 > Mc3 > Mc4 > Mc5$ (78) (Table 1). However, $Mc2$ -r only binds ACTH (114). The $Mc2$ -r requires two peptide domains for recognition and activation, the core H-F-R-W sequence presented in all the melanocortin peptides and a highly basic motif found only in the midportion of ACTH (79). $Mc1$ -r also shows a preferential binding for α -MSH (115). In addition, γ -MSH is relatively more selective for $Mc3$ -r vs. $Mc4$ - and $Mc5$ -r (78,116). Further, $Mc3$ -r and $Mc4$ -r are activated by the predominant form of α -MSH found in the periphery (monoacetyl- α -MSH) as well as the brain (desacetyl- α -MSH) (78,79), although the potency of diacetyl- α -MSH to activate $Mc4$ -r is much lower than that of desacetyl- α -MSH and α -MSH (79) (Table 1). However, the physiological relevance of these observations is not clear.

Melanocortins exert pleiotropic functions through their action on different melanocortin receptor subtypes. These functions include both peripheral and central effects such as regulation of pigmentation ($Mc1$ -r), lipolysis ($Mc2$ -r), adrenal steroidogenesis ($Mc2$ -r), anti-inflammation and anti-pyretic effect, grooming, regulation of cardiovascular function, improvement of memory, and regulation of energy balance (78, 116).

2. Mechanism of agouti in regulating pigmentation

Both eumelanin and phaeomelanin are derived from tyrosine, which is converted to dopa (3,4-dihydroxyphenylalanine) and subsequently to dopaquinone by the action of tyrosinase, the rate-limiting enzyme for both eumelanin and phaeomelanin synthesis (117). Under differential cellular environments, dopaquinone may diverge to form either eumelanin or phaeomelanin (117-120). Kobayashi et al. reported that while the expression and enzyme activity of tyrosinase was reduced in follicular melanocytes of lethal yellow mice and of 5- to 7-day-old agouti mice, that of tyrosinase-related protein 1 (TRP 1) and 2 (TRP 2) was completely inhibited (121). This is consistent with the fact that tyrosinase is required for both types of melanin synthesis, whereas TRP 1 and 2 are required only for eumelanin synthesis (122-124). In addition, differential display studies indicates that three novel genes are upregulated and four additional genes besides tyrosinase and TRP 2 are downregulated during phaeomelanin synthesis (125).

The relative amount of eumelanin and phaeomelanin is controlled by two loci in mammals, the *extension* locus, which encodes the Mc1-r (126), and the *agouti* locus. In the absence of agouti, α -MSH binding to Mc1-r results in an activation of adenylate cyclase and an increase in cyclic AMP levels (127), leading to upregulation of tyrosinase and other enzymes involved in melanogenesis, and an increase in eumelanin and total melanin synthesis (127). This has been demonstrated both in mouse (128) and human (129) melanocytes. In addition, variants of the Mc1-r are also found in humans (79,130), resulting in red hair and fair skin (130).

Earlier studies showed that injection of α -MSH into dominant yellow agouti mutants overrides agouti's action and produces a darkening coat; however, yellow recessive

extension mutants, which lacks the functional Mc1-r, are resistant to α -MSH treatment (131). This observation suggests that agouti may act as an antagonist of α -MSH. More recently, it was reported that recombinant murine agouti protein is a competitive antagonist of α -MSH (63, 132,133). Agouti protein inhibits α -MSH binding to Mc1-r and blocks α -MSH stimulation of adenylate cyclase, leading to a decrease in cAMP levels (63,132,133) in both murine melanoma cell line (132,133) and human embryonic kidney 293 cells stably transfected with Mc1-r (63). In addition, murine agouti protein has been shown to potently antagonize another melanocortin receptor, Mc4-r (63); however, it does not antagonize rat Mc3-r or mouse Mc5-r (63).

The human agouti protein, also called agouti signaling protein (ASIP), has been demonstrated to inhibit cAMP production stimulated by α -MSH in cells transfected with human Mc1, Mc3, Mc4 and Mc5 receptor and by ACTH in cells transfected with human Mc2r (134). ASIP also inhibited binding of radiolabeled α -MSH and ACTH to hMCR 1-5 (134). However, while ASIP is a potent inhibitor of the hMc1-r, Mc2-r and Mc4r, it has relatively weak effects at the hMc3-r and hMc5-r (134). In addition, the mechanism of ASIP antagonism varies among receptor subtypes, with characteristics consistent with competitive antagonism observed only at the hMc1r, and more complex behavior observed at the other receptors (134).

It is well documented that both murine and human agouti inhibit α -MSH stimulated melanogenesis in melanocytes (135-139). However, a substantial body of evidence also demonstrated actions of agouti protein on melanocytes that is independent of α -MSH (135-139). It is reported that both murine agouti protein and human ASIP inhibits the

expression and biosynthesis of tyrosinase, TRP 1 and 2, adenylylase and melanogenesis in murine and human melanocytes in the absence of α -MSH (135-139). In addition, while some found that agouti does not inhibit forskolin (an adenylylase activator) stimulation of adenylylase activity and dibutyryl cAMP stimulation of melanogenesis (138), others found that agouti also inhibits the action of forskolin, cholera toxin and pertussis toxin in melanocytes (135, 136, 139). It is noteworthy that α -MSH treatment results in an increase in both eumelanin and pheomelanin synthesis and an increase in eumelanin-to-pheomelanin ratio (138), while agouti treatment results in a decrease in both pigment production and a decrease in eumelanin-to-pheomelanin ratio (138). This is consistent with the fact that α -MSH and agouti result in a stimulation and inhibition, respectively, of tyrosinase, which is required for both eumelanin and pheomelanin synthesis.

It is hypothesized that agouti may act at a different receptor. However, substantial evidence demonstrates that this is not the case. Agouti protein specifically competes with radiolabeled melanocortins binding to melanocortin receptors (63, 132-134). In addition, several studies showed that both the inhibitory actions of agouti on α -MSH and the independent actions of agouti are dependent on the presence of the Mc1 receptor (131, 138, 139). Using epitope-tagged Mc1-r and agouti, Ollmann et al. also demonstrated that only cells expressing Mc1-r binds agouti (140). Further, although mice with loss of Mc1-r mutation have a similar yellow coat as dominant agouti mutants, these two coat colors are still different (140). This suggests that dominant yellow agouti animals are not equivalent to a simple loss of function of Mc1-r, and the action of agouti is more than simply blocking Mc1-r activity. However, Ollmann et al. demonstrated that the coat color

of the double mutants is the same as loss of Mc1-r mutants (140), demonstrates that Mc1-r is required for agouti action *in vivo* in coat color regulation.

Alternatively, Siegrist et al. proposed that agouti may act as an inverse agonist on Mc1-r thereby interfering with a certain level of constitutive Mc1-r activation present in the absence of MSH (139). It is also possible that agouti may interact with the Mc1-r to affect some other second messenger system (138), which would explain the inhibitory effect of agouti on forskolin, cholera toxin and pertussis toxin that are downstream of melanocortin receptors. Increasing intracellular calcium ($[Ca^{2+}]_i$) has been shown to inhibit melanogenesis (137,141). In addition, several studies demonstrated that agouti protein stimulates an influx of calcium in several cell types (142,143), and that agouti protein exerts pleiotropic action in adipose tissue via a Ca^{2+} -dependent mechanism which may be implied in agouti's action in obesity (23, discussed below).

Finally, agouti protein causes a downregulation of Mc1-r during long term treatment (139); however, it does not affect Mc1-r mRNA level (135).

3. Role of agouti in obesity

a. Melanocortin receptors and central effect

Agouti antagonism of Mc1-r does not contribute to agouti-induced obesity. Mice carrying recessive mutations at the *extension* locus have yellow coat but do not become obese (144). In addition, A^y mice carrying the dominant black mutation E^{so} , which has a constitutive active Mc1-r, are black and become obese (145). These observations suggest that the two phenotypes are separable and that agouti antagonism of Mc1-r and subsequent alterations of pigmentation is not involved in agouti induced obesity.

Alternatively, overexpression of agouti in novel tissues may lead to agouti antagonism of other melanocortin receptors, and thereby contribute to agouti induced obesity. Both Mc3-r and Mc4-r are expressed in hypothalamus including ventromedial (VMH) and arcuate (ARC) nuclei (146), brain regions that plays a key role in the regulation of feeding behavior. In addition, altered energy balance (food restriction-induced weight loss and diet-induced obesity) in rats causes changes in Mc4-r density in specific hypothalamic regions while that of Mc3-r remains unchanged (146), indicating an important role for Mc4-r in regulating feeding. Both murine and human agouti proteins are potent antagonists to the mouse and human Mc4-r, respectively. Recent research showed that Mc4-r knockout mice developed an obesity syndrome associated with hyperphagia, hyperinsulinemia and insulin resistance, similar to that found in agouti mutants (147). In addition, intracerebroventricular administration of the Mc4-r agonist, MTII, inhibited feeding, whereas co-administration of the Mc4-r antagonist, SHU9119, completely blocked this effect (148). Administration of SHU9119 alone also significantly enhanced feeding (148). These data indicate that chronic disruption of Mc4-r may be responsible for agouti induced obesity.

Many studies suggest that Mc4-r is also involved in human obesity. Chagnon et al. demonstrates a significant association between restriction fragment polymorphisms of Mc4-r and obesity phenotypes including body mass index (BMI), fat mass, percent body fat in the Quebec family study (82). Further, mutations in Mc4-r, although rare, have been identified in cases of human obesity (149-153), suggesting the significance of Mc4-r signaling pathway in the regulation of body weight.

The endogenous ligands for Mc4-r are melanocortin peptides derived from POMC. Yaswen et al demonstrated that mice lacking POMC developed severe obesity, altered pigmentation and defective adrenal development, while daily intraperitoneal administration of a α -MSH analog for 2-week results in a significant weight loss (154). Similarly, cases of severe obesity, adrenal insufficiency and red hair pigmentation caused by POMC mutations are identified in humans (155).

Agouti-related protein (Agrp) was recently identified by sequence similarity search using a characteristic pattern of cysteine spacing from the carboxyl terminal region of agouti (156, 157). Agrp encodes a 132-amino acid polypeptide with an N-terminal signal peptide, similar to that of agouti (157). It exhibits 25 % identity to agouti, with the highest degree of identity in the cystein-rich C-terminus (157). Agrp is expressed in the hypothalamus and adrenal gland of normal mice and humans (156, 157). Further studies demonstrated that Agrp mRNA is found in the arcuate nucleus of the rat, and Agrp fibers are found in a wide area of hypothalamus, including areas along the third ventricle, the periventricular nucleus (PVN) and the dorsomedial nucleus (DMH) (158). Agrp expression is upregulated in obese mice models such as *ob/ob* and *db/db* (156, 157). However, it is downregulated in the hypothalamus of *A^y* animals (156).

Biochemical studies demonstrate that Agrp is a potent antagonist to Mc3-r and Mc4-r, similar to agouti, but only has little effect on Mc5-r and no effect on Mc1-r and Mc2-r in terms of both cAMP production and radiolabeled ligand binding (156, 159). Transgenic mice expressing the human Agrp under the control of β -actin exhibit 70-100 % increases in body weight, similar to that of β -actin-agouti transgenic animals (156). Body length and food consumption was also increased by the Agrp transgene (156).

However, *Agrp* transgenic mice do not exhibit changes in coat color (156), suggesting that *Agrp* does not antagonize *Mc1-r*. In addition, intracerebroventricular (ICV) administration of *Agrp* increased feeding in rats; it also reversed α -MSH inhibition of feeding in these animals (160). These observations demonstrate that *Agrp* is the endogenous antagonist of the hypothalamic *Mc4-r*, which may be involved in the regulation of food intake.

Several lines of evidence indicate that *Agrp* and *Mc4-r* function downstream of leptin in the hypothalamus. Leptin is the product of the *ob* gene (161). It is produced primarily by adipose tissue (41) and conveys information about the body's fat reserves and energy state to the hypothalamus, leading to altered food intake and energy expenditure.

The functional long form leptin receptor is expressed in *Agrp*- and POMC-producing neurons in the hypothalamic arcuate nucleus (162-164), and the expression of *Agrp* and *Mc4-r* signaling appears to be regulated by leptin. Hypothalamic *Agrp* expression is elevated in leptin-deficient *ob/ob* mice and leptin-resistant *db/db* mice (156,157, 165) and by fasting, which is associated with a decline in circulating leptin levels in normal mice (162,165). In addition, leptin injection in *ob/ob* mice and normal mice resulted in significant decrease in *Agrp* mRNA level (162,165). The change in *Agrp* mRNA levels correlates with the change in neuropeptide Y (NPY), a potent orexigenic peptide, mRNA levels (165). In addition, double-staining study demonstrates that *Agrp* co-exists with NPY in the same cell bodies in the arcuate nucleus (166,167). These observations indicate that *Agrp* is regulated similarly to that of NPY in response to leptin. POMC expression is significantly reduced in *ob/ob* mice and *db/db* mice and by fasting

in normal mice (168-170), while it is upregulated by leptin treatment (168-170).

Pharmacological studies also suggest that part of leptin's effect is mediated by Agrp and Mc4-r signaling pathway. ICV injection of the Mc4-r antagonist, SHU9119, or Agrp markedly blocked leptin-induced anorexia in rats (160,171). However, recent studies showed that obese Mc4-r deficient mice do not respond significantly to the inhibitory effects of leptin on feeding, whereas non-obese young Mc4-r-deficient mice do (172). These observations demonstrate that Mc4-r is not an exclusive target of leptin action. Indeed, there is evidence that, in addition to Mc4-r, galanin, melanin-concentrating hormone (MCH), neurotensin (NT) and NPY are also targets of leptin signaling in the hypothalamus (173). In addition, recently identified neuropeptides orexins and cocaine- and amphetamine-regulated transcript (Cart) have been shown to be regulated by leptin (174-176).

Plasma leptin levels are elevated in yellow A^y/a mice as well as in β -actin-agouti overexpressing transgenic mice (42, 74). Interestingly, recent studies demonstrated that agouti protein directly stimulates leptin expression and secretion in both 3T3-L1 murine adipocytes and human adipose tissue, which is independent of Mc4-r (74). Up-regulation of leptin production by agouti may serve as a counterregulatory mechanism to attenuate agouti-induced obesity. However, yellow A^y/a mice fail to respond to exogenous leptin administration (44), indicating a leptin-resistance in these animals.

b. Peripheral actions of agouti

Recent studies demonstrated that agouti protein also exhibits potent pleiotropic effects in peripheral tissues. Adipose tissue expression of fatty acid synthase (FAS), the key enzyme in *de novo* lipogenesis, is significantly elevated in yellow obese mice (23). In

addition, recombinant agouti protein up-regulates FAS in both human adipose tissue and 3T3-L1 murine adipocytes (23). Agouti regulation of adipocyte FAS expression is mediated via the stimulation of FAS transcription rate and an agouti-response element has been identified in FAS promoter (177).

Mynatt et al. reported that transgenic mice specifically overexpressing agouti in adipose tissue under the control of aP2 promoter on a Purina Mouse Diet 5015 (11 % fat by weight) did not exhibit increases in body weight (178). However, subsequent examination revealed increases in fat pad mass in these aP2-agouti transgenic mice compared to non-transgenic controls (178-180). Moreover, concomitant hyperinsulinemia induced by daily insulin injection resulted in significant increases in body weight in aP2-agouti transgenic mice but not in their non-transgenic littermates (178). Follow-up studies found that on either a AIN93-G diet, which contains 17 % of fat, or a high sucrose-high fat diet, these aP2-agouti transgenic mice do have significantly greater body weight, fat pad mass and concomitantly higher leptin expression on either diet compared to nontransgenic littermates (179). In addition, inducing hyperinsulinemia in these aP2-agouti transgenic animals with high-sucrose feeding also results in significant obesity (180). Comparison of adipose tissues of transgenic to that of wild-type mice revealed that genes involved in insulin signaling are up-regulated in transgenic mice (179). These observations suggest that agouti expression in adipose tissue results in a great increase in energy partitioning into adipose tissue, leading to accretion of adipose tissue mass (179). In addition, there is an interaction between hyperinsulinemia and adipose tissue specific agouti expression in agouti-induced obesity syndrome (178-180).

Melanocortin receptors are also expressed in both human adipose tissue and 3T3-L1 adipocytes (82,84), and agouti acts as a paracrine factor that does not enter the circulation (30). Consequently, it is possible that agouti may exert its effect in adipose tissue via adipocyte melanocortin receptors in a paracrine/autocrine manner. Alternatively, the C-terminal region of agouti protein is highly homologous to that of neurotoxins (conotoxins and plectoxins) that primarily target calcium channels (63,64). Indeed, agouti stimulates significant Ca^{2+} influxes in several cell types, including adipocytes, leading to an increase in intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) (142,143). In addition, agouti protein up-regulates adipocyte FAS via a Ca^{2+} -dependent mechanism (23,181), and the agouti-response element in FAS promoter (177) also responds to KCl, which increases $[\text{Ca}^{2+}]_i$ by depolarization (unpublished observations). However, agouti only induce increases in $[\text{Ca}^{2+}]_i$ in cells transfected with melanocortin receptors (143), indicating that agouti's action on adipocyte calcium signaling also requires a functional melanocortin receptor. However, this is not mediated via melanocortin antagonism, as the effect persists even in the presence of high doses of a potent melanocortin agonist, Nle^4 , D-phe^7 - α -MSH (NDP- α -MSH) (143). Recent studies showed that administration of NDP-MSH to either yellow obese A^y mice or β -actin-agouti transgenic mice partially reversed coat color abnormalities but did not reverse any of the metabolic parameters in these obese animals (182), indicating that melanocortin antagonism may not explain all the characteristics in yellow obese mice. This is in clear contrast to central effects of agouti, which are dependent upon Mc4-r/Mc3-r antagonism to induce hyperphagia and obesity. Interestingly, feeding β -actin-agouti transgenic mice the calcium channel antagonist nifedipine results in decrease in fat pad mass, adipose tissue FAS activity, transient

increase in core temperature, normalization of plasma insulin levels and improvement of insulin sensitivity (183). These observations indicate that $[Ca^{2+}]_i$ may play an important role in agouti-induced obesity.

c. The mouse *mahogany* and *mahoganoid*

The mouse mutations *mahogany* (*mg*) and *mahoganoid* (*md*) are negative modifiers of the *agouti* coat color gene (184). Mice mutant for *mg* and *md* suppress the effects of *A^y* on both coat color and obesity (184,185). Genetic studies demonstrate that *mg* and *md* act along the agouti-melanocortin pathway, probably downstream of agouti but upstream of Mc1-r, as a loss-of-function Mc1r mutation (*recessive yellow*, *Mc1r^e*) suppressed the effects of *md* and *mg*, while *md* and *mg* suppressed that of *A^y* (184). Further studies demonstrate that the effect of *mg* is specific for agouti, as mutations of *mahogany* do not suppress weight gain in other obese animal models such as *ob/ob*, *db/db*, *tub/tub* and *Cpe^{fat}/Cpe^{fat}* (185,186). Mutation at *mg* also does not reverse obesity in Mc4-r knock-out animals, further confirming that *mg* acts at or upstream of melanocortin receptors (186). However, mutations at *mg* reversed diet-induced obesity (186). Indeed, *mg* mice eat more than normal mice; however, they do not gain weight, presumably because they have an increased basal metabolic rate and increased motor activity independent of agouti mutation (185). This may also explain the fact that *mg* mice are resistant to diet-induced obesity. Mutations of *mg* do not interfere with melanocortin production, as plasma α -MSH and ACTH levels are unaffected by *mg* or *md* (184). In addition, *mg* does not interfere with the secretion, processing or stability of agouti protein, as Western analysis detected no changes in either the mobility or the intensity of agouti protein from

hypothalamus of *A^{y/a}*, *mg/mg* mice (187). These observations suggest that *mg* mutation may interfere with agouti signaling (184, 187).

The gene encoding *mg* has been cloned (186,187) and shown to encode a 1,428-amino acid, single-transmembrane-domain protein that is expressed in many tissues, including pigment cells and the hypothalamus (186,187). The cytoplasmic tail of mahogany is short ($\approx$ 128 amino acids) and has no previously defined signaling domain (186,187). However, the large extracellular domain is the orthologue of human attractin, a circulating molecule produced by activated T cells that has been implicated in immune-cell interactions (187,188). The extracellular domain contains several epidermal growth factor (EGF) domains, a CUB domain, a C-type lectin domain and several potential glycosaminoglycan attachment sites (187-189). The EGF domain is frequently found in proteins that interact with other proteins that are involved in extracellular signaling or cell guidance (188), while the CUB and C-type lectin domains suggest that the protein interacts with carbohydrate moieties (189). It has been hypothesized that mahogany may serve as a low affinity receptor for agouti, thereby increases its local cell-surface concentration (186,187). The N-terminal domain of agouti is glycosylated and very basic, and could interact with the C-type lectin domain and/or potential glycosaminoglycan side chains of mahogany (187). Indeed, Barsh et al demonstrated that mahogany binds recombinant agouti protein but not *Agrp* (190), which has a homologous C-terminal region but an N-terminal region that is largely different from agouti (157). In addition, transgenic mice overexpressing an agouti mutant lacking the entire basic region (Lys57-Arg85) do not become obese, although the level of the transgene expression is comparable to or higher than that in obese viable yellow mice (191), further confirming

the importance of the N-terminal region in agouti-induced obesity. The discovery of mahogany adds more complexity to agouti's action, and may provide new insight into the regulation of energy metabolism and body weight.

d. Structure-function relationship of agouti protein

Transgenic studies demonstrated that overexpressing agouti *per se* is responsible for the pleiotropic obesity syndrome in yellow mice, as transgenic mice overexpressing agouti under the control of β -actin promoter recapitulated the yellow obese syndrome (73). Additional mutagenesis and transgenesis studies demonstrated the structure-function relationship in agouti protein. Agouti C-terminal fragment (Val83-Cys131) is equally as potent as full-length agouti in melanocortin receptor antagonism (63). Mutations of several residues at the C-terminal of agouti decrease its inhibition of ligand binding to the melanocortin receptor (192). This study revealed that Val 83 is important for inhibition of melanocortins binding to Mc1-r while Arg85, Pro86 and Pro89 are important for selective inhibition of melanocortins binding to Mc3-r and Mc4-r (192). There are 10 cysteine residues in the C-terminus of agouti protein, which form 5-disulfide bonds (63,64). The cysteine residues in conotoxins and plectoxins, which bear structural similarity to that of agouti, are critical for toxin activity as they stabilize the tertiary structure of the toxin through the formation of disulfide bonds (193). Mutagenesis /transgenesis studies revealed that individual substitution of 8 out of 10 cysteine residues with serine results in a complete loss of agouti activity on both coat color and obesity, while individual substitution of the other 2 cysteine residues with serine results in a partial loss of agouti activity (194). Transgenic mice overexpressing agouti with a point mutation or a 10-amino acid-deletion in the signal sequence results in a complete loss of

agouti activity, due to the interference with its secretion (194, 195); these mice were neither yellow (194,195) nor obese (194). Mutation at Asn 39, the potential N-glycosylation site, partially impaired agouti activity such that these mice developed a partially yellow color, but they are neither completely yellow nor do they become obese (194). Deletion of half of the central basic region of agouti (Arg64-Lys77) had no apparent effect on agouti activity (194). However, several basic amino acids remain intact both N- and C-terminal to this deletion (191). Indeed, deletion of the entire central basic region of agouti (Lys57-Arg85) reduces agouti antagonism of melanocortin receptors by about 10-fold in vitro (192). In addition, transgenic mice overexpressing agouti with deletion of this basic region (Lys57-Arg85) under the control of β -actin promoter developed partially yellow fur but not obesity (191).

e. The human homolog of agouti

The human homologue of agouti (ASIP) is expressed in adipose tissue as well as in other tissues (60, 61). Although several linkage analysis failed to find an association between human obesity and ASIP, Norman et al did demonstrate a linkage between a loci on human chromosome 20q11.2, where the human agouti gene is localized, and 24hr respiratory quote (24RQ) in Pima Indians (196). In addition, recent studies discovered restriction fragment-length polymorphism (RFLP) for Mc4-r and Mc5-r, which are localized on human chromosome 18p, and linkages between Mc4-r and Mc5-r and obesity-related phenotypes (82).

III. OBESITY AND SYNDROME X: ROLE OF INTRACELLULAR CALCIUM

Obesity is closely related to other metabolic disorders, including non-insulin-dependent diabetes mellitus (NIDDM), hypertension and cardiovascular disease, all of which are accompanied by hyperinsulinemia and insulin resistance. These diseases are parts of an integrated metabolic syndrome referred to as "Syndrome X" (52). Based on numerous observations of cellular ionic lesions in these diseases, Resnick et al proposed a unifying "ionic hypothesis", in which a common cell lesion underlying these disorders in different tissues was characterized, in part, by elevations in steady-state $[Ca^{2+}]_i$ levels (197,198). Sustained increase in $[Ca^{2+}]_i$ levels have been observed in vascular smooth muscle cells, platelets, lymphocytes, aorta, renal cells and erythrocytes either in patients with hypertension or in hypertensive rats (199-204) and skeletal muscle cells, erythrocytes, platelets and adipocytes in patients with obesity and/or insulin resistance (205-209). $[Ca^{2+}]_i$ levels are also increased in platelets in elderly people, as aging is associated with alterations in insulin sensitivity, blood pressure and adiposity (210). In addition, positive correlations have been found between $[Ca^{2+}]_i$ levels and BMI, diastolic blood pressure and fasting blood glucose levels (207,208). Further, weight reduction resulted in a decrease in $[Ca^{2+}]_i$ levels in obese and mildly hypertensive people (211).

Obesity is also associated with alterations in calcium homeostasis. In normal plasma, approximately 50 % of total calcium are ionized, 35 % are protein bound, which mostly is albumin-bound, and 15 % is complexed to small anions. Only the ionized calcium is physiologically active, and is regulated by parathyroid hormone, 1,25-dihydroxyvitamin D and calcitonin. Normal serum total calcium level is around 2.5 mM, leaving serum ionized Ca^{2+} level at around 1.25 mM. Decreased serum ionized Ca^{2+} ,

increased urinary calcium excretion, and increases in 1,25-dihydroxyvitamin D and parathyroid hormone (PTH) have been frequently observed in obese individuals (212-215). Serum total calcium levels are usually unchanged (212-215). However, Andersen et al reported that serum albumin, phosphate and bicarbonate are decreased in obesity, while the concentrations of citrate, lactate, acetoacetate, 3-hydroxybutyrate, free fatty acids and urate are all increased (213). These changes increase the concentrations of less readily reabsorbable anion in the renal ultrafiltrate, which would in part explain the observations of increased urinary calcium excretion and decreased serum ionized Ca^{2+} (216, 217), leading to elevations in serum calcitropic hormones (213). An inverse correlation has been found between serum ionized Ca^{2+} and body mass index (BMI) and % of overweight in these studies (213, 218). In addition, weight reduction results in normalization of serum phosphate, bicarbonate, lactate and free fatty acids level, leading to increases in serum ionized Ca^{2+} levels and a reduction in calcitropic hormones (219).

Calcitropic hormones such as 1,25-dihydroxyvitamin D has been shown to stimulate Ca^{2+} influx in both vascular smooth muscle cells and pancreatic β -cells, indicating that 1,25-dihydroxyvitamin D, elevated with the disturbance of calcium homeostasis commonly seen in these diseases, may also play a role in the development and further promotion of hypertension and hyperinsulinemia, respectively (220,221). In addition, 1,25-dihydroxyvitamin D has been demonstrated to act on human adipocytes to cause a sustained increase in $[\text{Ca}^{2+}]_i$ and a corresponding marked inhibition of lipolysis (222), suggesting that increased 1,25-dihydroxyvitamin D in obesity may also play a role in the development and promotion of obesity.

Insulin has been shown to attenuate both voltage- and receptor-mediated Ca^{2+} influx in cultured rat vascular smooth muscle cells (223). In addition, insulin has been shown to attenuate phenylephrine, a potent α -adrenergic agonist and vasoconstrictor, induced increases in $[\text{Ca}^{2+}]_i$ in rat adipocytes (224). Recent studies showed that insulin stimulates Ca^{2+} -ATPase activity in a variety of tissues (225), and Ca^{2+} -ATPase activity is reduced in type II diabetes (226,227). Further, vascular smooth muscle cell Ca^{2+} efflux is also impaired in both insulin resistant (Zucker obese) and insulinopenic (streptozotocin-treated) rats (228-230). This effect is exerted via the upregulation of Ca^{2+} -ATPase expression, as reduced Ca^{2+} -ATPase expression has been observed in vascular smooth muscle from Zucker obese rats (231) and insulin directly stimulates vascular smooth muscle cell Ca^{2+} -ATPase expression (232). Thus, it is possible that in obesity, diabetes and hypertension, which are usually accompanied with hyperinsulinemia and insulin resistance, a reduction in Ca^{2+} -ATPase expression and activity would result in a decrease in Ca^{2+} efflux, which contribute to the increased $[\text{Ca}^{2+}]_i$ in these disease status.

In addition, thiazolidinedione derivatives, such as troglitazone, ciglitazone and pioglitazone, which have been shown to increase insulin sensitivity, reduce blood glucose, insulin and lipid levels and lower blood pressure (233), have been demonstrated to inhibit calcium channel activity and K^+ -induced contraction in smooth muscle cells from resistance arteries of guinea-pig (234), and to inhibit platelet-derived growth factor-induced increases in intracellular free calcium in human glioblastoma cells and rat vascular smooth muscle cells in vitro (235).

$[\text{Ca}^{2+}]_i$ appears to be involved in the regulation of adipocyte lipid metabolism. Avasthy et al reported that the calcium ionophore A23187 augmented both the basal and

insulin-stimulated lipogenesis in rat adipocytes while the calcium channel antagonist verapamil as well as calmodulin inhibitor calmidazolium inhibited both basal and insulin-stimulated lipogenesis (236).

In addition, the role of $[Ca^{2+}]_i$ in the regulation of adipocyte lipid metabolism has been studied in yellow obese agouti mutants. A^{vy} mice exhibit increases in both steady state $[Ca^{2+}]_i$ and Ca^{2+} influx in several tissues (142,143). The increase in $[Ca^{2+}]_i$ is correlated with both the degree of ectopic agouti expression and body weight (142). In addition, A^{vy} mice exhibit elevated adipocyte fatty acid synthase (FAS) expression, suggesting that adipocyte *de novo* lipogenesis is increased in these animals (23). Further, agouti protein promotes adipocyte triacylglycerol content by upregulating adipocyte FAS, a key enzyme in *de novo* lipogenesis (23), as recombinant agouti protein stimulates FAS expression and activity in 3T3-L1 adipocytes as well as in human adipocytes (23,177). This effect is mediated by $[Ca^{2+}]_i$, as agouti's effect can be inhibited by the calcium channel antagonist nitrendipine (23), and recapitulated by calcium channel agonist (237,238).

Draznin et al showed that maximum insulin-stimulated glucose transport in rat adipocytes is achieved at extracellular calcium concentrations of 1 and 2 mM and intracellular calcium concentrations ranging from 140 to 370 nM, with elevations or reductions beyond these concentrations causing diminished cellular responsiveness to insulin (239). Similarly, an optimal extracellular calcium concentration has been demonstrated in adipocyte lipolysis (240). Both basal and hormone-stimulated lipolysis have been shown to be optimal at extracellular calcium concentration of 0.925 mM, close to that of normal serum ionised Ca^{2+} , while lipolysis is markedly decreased at

extracellular calcium concentrations of either 0 or 2.77 mM (240). This indicates a conditional effect of extracellular calcium in cellular functions.

In addition, both depleting $[Ca^{2+}]_i$ with EGTA and increasing $[Ca^{2+}]_i$ with A23187 inhibits adipocyte lipolysis (241). This suggests that an optimal range of $[Ca^{2+}]_i$ is also exist for lipolysis. The inhibitory effect of $[Ca^{2+}]_i$ on adipocyte lipolysis has been reported in several studies (241-244). However, the mechanism(s) mediating this effect remains unclear. Studies with epidermal growth factor (EGF) shows that the anti-lipolytic effect of EGF, which is mediated through an increase in $[Ca^{2+}]_i$, primarily involves the modulation of the interaction between G_i and adenylate cyclase, leading to a reduction in cAMP (244,245). However, studies of the lipolytic response in hypothyroid rats indicate that thyroid hormone status modulates adipocyte lipolysis by alterations in cellular Ca^{2+} concentrations that involve alterations in phosphodiesterase activity (243). In addition, increasing $[Ca^{2+}]_i$ by the calcium ionophore A23187 in hamster adipocytes results in stimulation of phosphodiesterase and a decrease in cAMP levels (242).

Increases in dietary calcium intake is inversely correlated to % body fat (222), hypertension (246) and NIDDM (247). One underlying mechanism is that increases in dietary calcium intake may suppress endogenous calcitropic hormones, leading to the decrease in intracellular calcium levels. This has been demonstrated by Sanchez et al (248), who reported that dietary calcium supplement resulted in significant reductions in serum osteocalcin, parathyroid hormone and 1,25-dihydroxyvitamin D levels, leading to a reduction in platelet intracellular free calcium levels, reduction in plasma insulin level and improvement of insulin sensitivity (248).

Previous data demonstrated that treating transgenic mice overexpressing agouti with a Ca^{2+} channel antagonist, nifedipine, results in significant decreases in adipocyte lipogenesis and reduction in adipose tissue mass (183). Similarly, Ca^{2+} channel antagonists, such as nifedipine, verapamil and felodipine, causes decreases in body weight and fat pad mass in obese SHHF/Mcc-fa^{cp} rats (249, 250).

IV. REGULATION OF LIPID METABOLISM IN ADIPOCYTES

A. Regulation of *de novo* lipogenesis in adipocytes

Lipogenesis refers to the conversion of glucose to triacylglycerol. The reactions of glycolysis and the citric acid cycle give rise to the major substrate for fatty acid synthesis: acetyl CoA. Acetyl CoA carboxylase (ACC) and fatty acid synthase (FAS) are two key enzymes involved in *de novo* lipogenesis. ACC catalyzes the rate-limiting step in lipogenesis, the conversion of acetyl CoA to malonyl CoA, while FAS catalyzes the subsequent synthesis of palmitate from acetyl CoA and malonyl CoA using NADPH as a reducing equivalent. ACC is subject to short-term regulation via allosteric and reversible phosphorylation event. However, long-term regulation of both ACC and FAS involves changes in the rate of enzyme synthesis (251).

Humans exhibit lower FAS activity in both liver and adipose tissue than that of rats (252), which can be attributed to the lower metabolic rate in humans than in rodents (252). Nonetheless, significant *de novo* lipogenesis is well documented in human adipocytes. Human adipocytes contain substantial levels of FAS expression and activity that are sensitive to both nutritional and hormonal modulation (253-255). Studies in humans given a high carbohydrate diet demonstrate that adipose tissue plays an important role in fat synthesis under these diets (256, 257). In addition, FAS levels are elevated in obesity (258). Thus, induction of adipocyte lipogenesis may contribute to obesity.

1. Fatty acid synthase

The fatty acid synthase from *E. coli* and higher plants consists of seven separate polypeptides that are tightly associated in a single, organized complex (259). However, FAS in vertebrates comprises a single large polypeptide (240 kDa). From its N-terminus

to its C-terminus, amino acids encoding β -ketoacyl synthase, acetyl-CoA transacylase, malonyl-CoA transacylase, dehydratase, enoyl reductase, ketoacyl reductase, acyl carrier protein and thioesterase are organized into discrete domains in the same order (260). Active FAS is a homodimer (480 kDa). The multifunctional organization of FAS probably is a result of gene fusion during evolution (261).

The synthesis of palmitate by FAS is a cyclic process in which an acetyl primer undergoes a series of decarboxylative condensation with seven malonyl moieties, leading to elongation of acyl group by two carbons per cycle yielding the saturated 16-carbon palmitate as the final product (261).

2. Regulation of fatty acid synthase by nutrients and hormones

Unlike ACC, FAS is not subject to allosteric regulation and post-translational modification. Rather, FAS is regulated mainly at the level of transcription (262).

FAS is very sensitive to changes in nutritional status. Fasting results in a significant decline of the rate of FAS synthesis in rats, while refeeding previously fasted animals a high carbohydrate diet increases the rate of FAS synthesis by 20-25 fold (263). In addition, Paulauskis et al reported that refeeding in mouse results in the increase in FAS gene transcription by up to 39-fold (264).

Carbohydrate plays an important role in regulating FAS. Experiments in hepatocytes and adipocytes demonstrate that glucose stimulates the transcription of lipogenic genes including FAS (265). However, 3-O-methylglucose, a glucose analogue transported into the cell but not phosphorylated by hexokinases, is unable to induce FAS expression in adipose tissue explants (266). 2-Deoxyglucose, which is transported into cells and phosphorylated to 2-deoxyglucose-6-phosphate (267), mimics the effect of glucose on

FAS expression (266). This suggests that glucose-6-phosphate and its metabolites may be the signal that mediates the effect of glucose in regulation of FAS (266).

On the other hand, dietary polyunsaturated fatty acid (PUFA) potentially inhibits FAS activity and expression. Dietary supplement of 2 % 18:2(n-6) to a high-carbohydrate, fat-free diet results in a marked suppression of hepatic fatty acid synthesis and the activities of FAS and other lipogenic enzymes (268), while saturated or mono-saturated fatty acids are without effect (268). This effect has been demonstrated to involve the dietary polyunsaturated fatty acids of the (n-3) and (n-6) families (269). PUFA not only inhibits FAS gene expression in adult rats, but also blocks the 20- to 30-fold rise in mRNA levels associated with weaning rats onto a high-carbohydrate diet (269).

It is widely accepted that circulating insulin levels are elevated by ingesting a high-carbohydrate diet, while circulating glucagon levels are elevated during starvation. The two hormones are also involved in the regulation of FAS. FAS mRNA levels are very low in streptozotocin-diabetic mice, but increase by 2-fold within 1 hr after insulin administration and 19-fold within 6 hrs after insulin administration (264). In contrast, administration of glucagon or dibutyryl cAMP during refeeding of previously fasting animals completely blocked the elevation of FAS transcription rates (264). These observations suggest that glucagon, via the elevation of cAMP, antagonizes insulin's effect on FAS regulation. Growth hormone also appears to antagonize the effect of insulin on FAS stimulation. Growth hormone decreases FAS mRNA level in rats and in cultured pig adipose tissue (270, 271).

Thyroid hormone (3,5,3'-Triiodothyronin, T₃) also stimulates FAS expression. Injection of thyroid hormone into rats for seven days increased FAS activity in liver

(272,273). In addition, both liver and white adipose tissue FAS mRNA expression is increased in hyperthyroid rats and decreased in hypothyroid animals (274).

In differentiated 3T3-L1 adipocytes, FAS expression is stimulated by insulin and T3 and inhibited by dibutyl cAMP (275,276). In addition, the synthesis rate of FAS in hepatocytes from mouse or rat is stimulated by insulin and T3 and inhibited by glucagon (277,278). The cis-acting insulin response element (IRE) has been identified in the region from -67 to -52 on the FAS promoter (279). Insulin also exerts modulatory effect on lipogenic enzymes in cultured human adipocytes. Treating primary cultured human adipocytes with insulin resulted in a dose-dependent increase in FAS activity (253).

In addition, Angiotensin II, which has been recently shown to be synthesized and secreted by adipocytes (280,281), stimulates FAS activity in both 3T3-L1 adipocytes and human adipose tissue (282). This effect is mediated at the transcriptional level via the IRE (283).

The human homologue of the mouse agouti, which is normally expressed in human adipose tissue (76), has been shown to upregulate adipocyte FAS activity and expression in both 3T3-L1 and human adipocytes (23,177). The effect of agouti is exerted via the stimulation of the rate of FAS gene transcription (177). In addition, the effects of agouti and insulin are independent and additive. Further studies demonstrated that the agouti response element is located in the -435 to -415 region of the FAS promoter, a region that is upstream of the insulin responsive element (177).

B. Regulation of lipolysis in adipocytes

1. Hormone sensitive lipase and adipocyte lipolysis

The quantitatively most important energy source in mammals is free fatty acids (FFA) derived from adipose tissue triacylglycerol (TAG), which accounts for > 95 % of body TAG storage (284). Released free fatty acids are transported in the bloodstream bound to albumin and taken up by other tissues. The small triglycerol stores of non-adipocytes most likely function as an internal energy reserve and as a supply of fatty acids for membrane biosynthesis (285).

Hormone sensitive lipase (HSL) plays a crucial role in the mobilization of free fatty acids from TAG in adipose tissue, a process referred to as lipolysis, and therefore is a key enzyme in lipid metabolism and overall energy homeostasis. It catalyzes the rate-limiting step in the hydrolysis of triacylglycerol to diacyl glycerol, and also the subsequent hydrolysis of diacylglycerol to monoacylglycerol. The last step in lipolysis, the conversion of monoacylglycerol to FFA and glycerol, is carried out by monoacylglycerol lipase (286). HSL is subject to acute hormonal and nutritional control; however, there is no evidence for any regulation of the monoacylglycerol lipase (287).

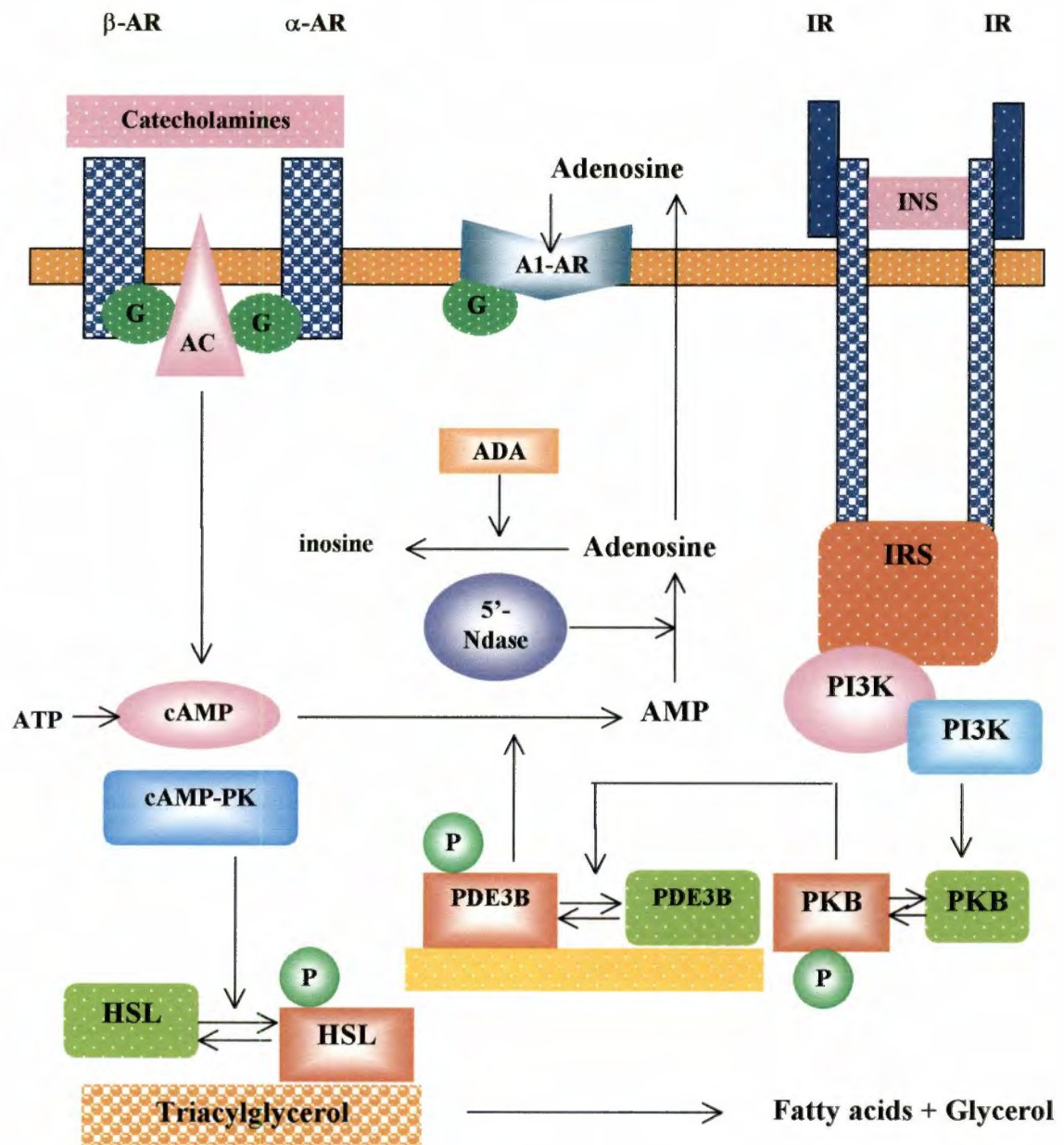
cDNA for HSL has been obtained from both rats and humans (288,289). The human HSL gene is composed of 9 exons, encoding a 775-amino acid protein (289). In addition to adipose tissue, HSL is also present in steroidogenic tissues such as adrenal cortex, ovaries and testis, as well as heart, muscle, pancreas, and macrophages (290-296). Regions upstream of exon 1 are expressed in a tissue-specific manner, which are either noncoding in adipose tissue or encode an N-terminal extension of the enzyme, as in testis (296) and in pancreas (295), leading to a relatively larger species in these tissues

(295,296). Consistent with its expression in steroidogenic tissues, HSL also has a significant activity against long chain esters of cholesterol (297), which is approximately equal to that against TAG, at least in vitro.

HSL protein is composed of an N-terminal domain and a C-terminal catalytic domain; the latter includes an active catalytic site, a regulatory module and a putative lipid-binding domain (298,299). Ser-423, Asp-703, and His-733 consist of the active catalytic triad of HSL (300,301). Although the function of the N-terminal domain in HSL is unclear, recent data showed that it is involved in the interaction of HSL with other cellular proteins, such as fatty acid binding protein (FABP) (302). From this point of view, it is interesting to notice that HSL species in the testis and pancreatic β -cells contains longer N-terminal domains, suggesting that the putative role of the extended N-terminus of HSL in testis and pancreatic β -cells may be involved in the interaction of HSL with other proteins.

Adipocyte lipolysis is under acute hormonal regulation (Figure 4). Lipolytic hormones, such as catecholamines, act through β -adrenergic receptors, resulting in increases in cAMP, which then activates cAMP dependent protein kinase A (PKA). The activation of PKA in turn phosphorylates and activates hormone-sensitive lipase. In contrast, insulin, acting through the insulin receptor and downstream phosphatidylinositol-3 (PI-3) kinase and possibly other downstream kinases, phosphorylates and activates a phosphodiesterase kinase, which is now believed to be protein kinase B (PKB) (303). PKB then phosphorylates and activates phosphodiesterase 3B (PDE 3B), a major isoform expressed in adipose tissue, which catalyzes the

Figure 4. Hormonal regulation of adipocyte lipolysis. Adipocyte lipolysis is under acute regulation. Lipolytic hormones, such as catecholamines, act through β -adrenergic receptors, resulting in increases in cAMP, which then activates cAMP dependent protein kinase A (PKA). The activation of PKA in turn phosphorylates and activates hormone-sensitive lipase. In contrast, insulin, acting through the insulin receptor and downstream phosphatidylinositol-3 (PI-3) kinase phosphorylates and activates protein kinase B (PKB). PKB then phosphorylates and activates phosphodiesterase 3B (PDE 3B), a major isoform expressed in adipose tissue, which catalyzes the degradation of cAMP, leading to a decrease in cAMP and PKA activity, and inhibition of lipolysis. α -AR: α -adrenergic receptor. β -AR: β -adrenergic receptor. AC: adenylate cyclase. cAMP: cyclic AMP. cAMP-PK: cAMP-dependent protein kinase A. HSL: hormone-sensitive lipase. A1-AR: A1 adenosine receptor. ADA: adenosine deaminase. 5'-Ndase: 5'-nucleotidase. IR: insulin receptor. INS: insulin. IRS: insulin-receptor substrate-1. PI3K: phosphatidylinositol-3 kinase. PKB: protein kinase B. PDE3B: phosphodiesterase 3B.



degradation of cAMP, leading to a decrease in cAMP and PKA activity, and inhibition of lipolysis (304,305).

HSL is regulated by reversible phosphorylation (Figure 5). It is phosphorylated at two distinct serine residues, termed the regulatory and basal site, respectively (306). PKA phosphorylates HSL at the regulatory site (Ser-563 and Ser-551 in rat and human HSL, respectively), leading to the activation of HSL (306-308). The basal site is only two amino acids away from the regulatory site, Ser-565 and Ser-553 in rat and human HSL, respectively. The basal site is phosphorylated by glycogen synthase kinase-4, Ca^{2+} /calmodulin dependent kinase II and 5'-AMP-dependent protein kinase (AMPK) (309,310). Phosphorylation of the basal site mainly occurs in resting cells and has been shown to exert an anti-lipolytic effect such that its phosphorylation prevents the phosphorylation of the regulatory site (311). Recently, two novel serine sites, Ser-659 and Ser-660 in rat HSL, has been identified to be phosphorylated both in vitro and in intact cells by PKA (312). These two sites have been demonstrated to be critical in regulating HSL activity as studied by site-directed mutagenesis (312), indicating that HSL is subject to complex phosphorylation regulation at multiple serine sites.

The mechanism of HSL activation is not clear. HSL is increased 2-3 fold by PKA in vitro, while a more than 20-fold increase in lipolytic rate occurred in intact fat cells in response to hormonal stimulation (304). This is in part explained by the translocation of HSL from cytosol to lipid droplets upon isoproterenol stimulation (313-315). Under resting status, HSL is mainly localized in cytosol. Upon lipolytic stimulation, HSL translocates from the cytosol to the lipid droplets (313-315). This is observed both in 3T3-L1 and rat adipocytes.

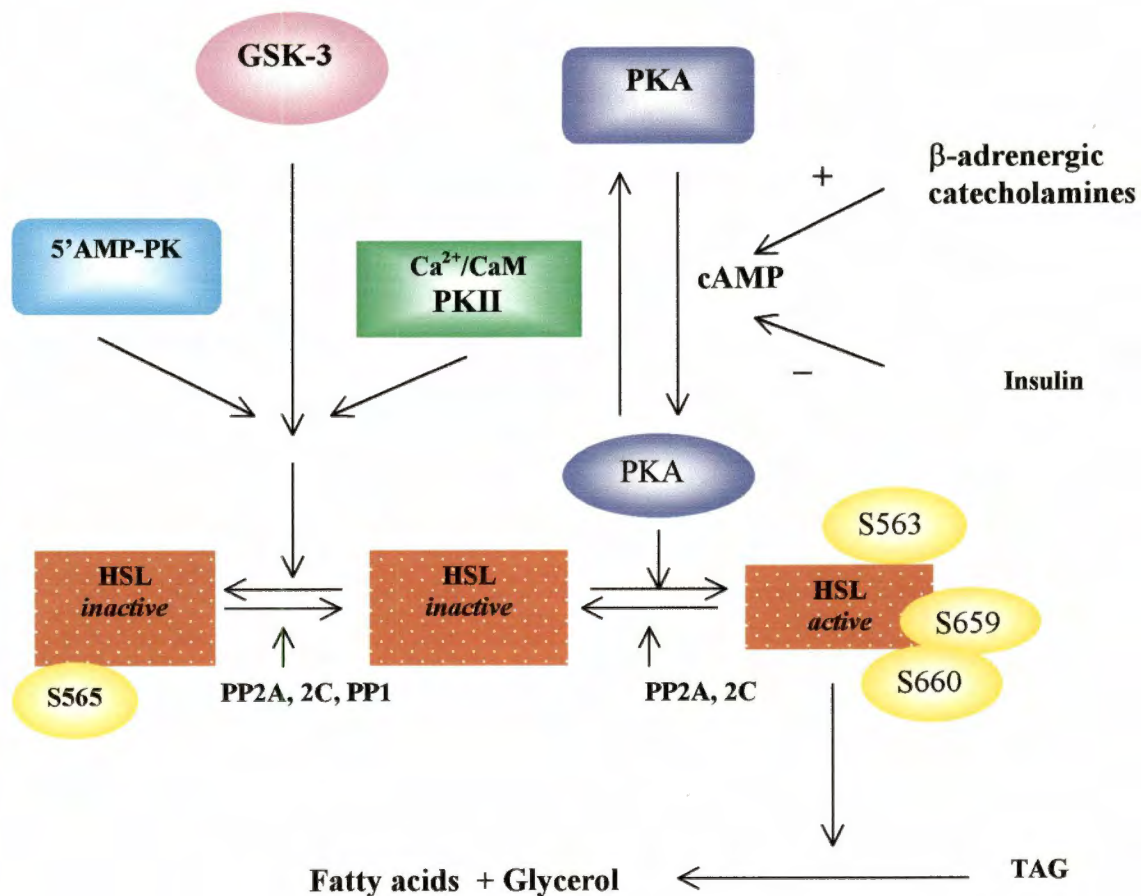


Figure 5. Regulation of hormone-sensitive lipase by reversible phosphorylation.

Activation of hormone sensitive lipase requires prior dephosphorylation of a basal site (Ser-565) and subsequent phosphorylation of a regulatory site (Ser-563) and additional serine residues (Ser-659 and Ser-660). GSK-3: glycogen synthase kinase-3. 5'AMP-PK: 5'AMP-dependent protein kinase. Ca²⁺/CaM PKII: Ca²⁺/calmodulin dependent protein kinase II. PKA: protein kinase A. HSL: hormone-sensitive lipase. PP1: protein phosphatase 1. PP2A: protein phosphatase 2A. PP2C: protein phosphatase 2C.

Perilipins, proteins found exclusively on the TAG-rich lipid droplets in adipocytes and the cholesterol ester-rich droplets in steroidogenic cells (316) are phosphorylated by PKA upon lipolytic stimulation (316). It is hypothesized that non-phosphorylated perilipin imposes a barrier to HSL actions that is attenuated by PKA phosphorylation (316). When perilipin constructs were transfected to cells that normally only have a small amount of lipid droplets, perilipin proteins localized to the lipid droplets and the intracellular TAG content increased as a result of decreased hydrolysis of lipids (316). This suggests a protective role of perilipins against lipid hydrolysis (316). In addition, tumor necrosis factor α (TNF- α) exerts potent lipolytic effect via the inhibition of perilipin expression, leading to a reduction of perilipin immunostaining at the droplet surface (316). However, overexpression of perilipin blocked TNF- α 's effect (317), further confirming the inhibitory role of non-phosphorylated perilipin on lipolysis. Phosphorylation of perilipin by PKA results in redistribution of perilipin on lipid droplets, leading to altered structure of the lipid droplet surface, which then allows HSL interacting with the core TAG in lipid droplets (316). Recently, a novel HSL-interacting protein, lipotransin, has been identified and appears to be involved in HSL translocation and interaction with lipid droplets (318). However, the exact function of lipotransin is not clear.

Overexpression of HSL in 3T3-L1 adipocytes or human hepatoma cell HepG2 results in diminished triacylglycerol accumulation (319,320), confirming the role of HSL as the key enzyme in lipolysis. However, mice homozygous for the null HSL allele are superficially normal except that the males are sterile because of oligospermia (321). These mice have normal gonadal and adrenal function, but the neutral cholesterol ester

hydrolase (NCEH) activity is completely lost in the testis, the white fat and brown fat. Both brown and white adipocytes are larger than those of wild type animals. However, although brown fat mass is increased by 1.65-fold, white fat mass remains unchanged and retained 40 % of triacylglycerol lipase activity. These mice were neither obese nor cold sensitive. These observations indicate that HSL may be critical in spermatogenesis in males, whereas redundant lipase activities may exist in other tissues, including adipocytes (321).

2. Nutritional and hormonal regulation of HSL

Nutritional status regulates lipolysis. Prolonged fasting for 3-5 days increases basal lipolysis rate, HSL activity, protein and mRNA level in adipocytes in rats (322). In addition, long-chain fatty acyl CoA and intermediary lipid metabolites, such as oleoyl CoA, palmitoyl CoA, oleic acid and 2-monopalitoylglycerol exert an inhibitory effect on HSL activity (323, 324), reflecting a feed-back inhibitory mechanism. Recent data showed that HSL interacts with adipocyte fatty acid binding protein (FABP) (302), which may serve to limit the inhibitory effect of intermediary lipid metabolites on HSL activity. The ketone bodies acetoacetate and β -hydroxybutyrate are potent inhibitors of lipolysis (325); these effects are demonstrable at plasma ketone body concentrations that occur after strenuous exercise, fasting and in uncontrolled diabetes, indicating that ketone bodies may be important modulators of lipolysis under these conditions (326).

Catecholamines are powerful regulators of lipolysis that are mediated via four adrenergic receptors: β 1, β 2, β 3, and α 2 (327). Signals through β receptors result in increases in cAMP and stimulation of lipolysis, while α 2 receptors have opposite effects on lipolysis. It is suggested that α 2 receptors predominate in the regulation of adipose

tissue lipolysis at rest and that β receptors assume a more important role during exercise (328). In addition, each receptor subtype may serve as a different signaling role. β_1 , which is more sensitive to catecholamines and desensitizes rapidly, may mediate acute effects of low level catecholamine stimulation, while β_3 receptors, which require higher levels of catecholamines to become activated and are more resistant to desensitization, may deliver a more sustained signal (327).

Growth hormone stimulates lipolysis in vitro and at physiological concentrations in vivo (329-331). Glucagon and the synthetic glucocorticoid dexamethasone also exert potent lipolytic effect in rat adipocytes (329). In addition, although epinephrine, glucagon and growth hormone do not affect the expression of HSL, dexamethasone increases both the lipolytic rate and HSL mRNA levels in rat adipocytes (329). In vivo, when compensatory hyperinsulinemia during cortisol infusion is prevented by a pancreatic clamp, cortisol infusion resulted in a 60 % increase in lipolysis in humans (332).

Tumor necrosis factor- α (TNF- α) is produced and secreted by adipose tissue, and its production is increased in obesity (333). Recent studies demonstrated that TNF- α is a potent lipolytic factor (334-336). This effect of TNF- α may in part contribute to the elevated blood free fatty acid levels frequently associated with obesity and diabetes, leading to insulin resistance (310).

Insulin is by far the most potent anti-lipolytic hormone and has been extensively studied. Recent studies demonstrated that lipolysis in normal subjects is exquisitely sensitive to insulin with a half-maximal effect (ED_{50}) occurring at a concentration of 12 pM (337). However, although it is well-documented that insulin acutely inhibits hormone-stimulated lipolysis, chronic exposure of rat fat cells to insulin resulted in

enhanced lipolysis and activation of hormone-sensitive lipase (338-339). This long-term effect of insulin may also contribute to the elevation of plasma free fatty acid levels in obesity and diabetes, which are frequently accompanied with hyperinsulinemia and insulin resistance. However, further studies are required to confirm these findings.

Adenosine is a potent anti-lipolytic factor (340) whose action is exerted through the A_1 adenosine receptor, which, by coupling to G_i , inhibits adenylate cyclase (341). Recent studies showed that cAMP is constantly exported from adipocytes to the extracellular space (342), and once exported, membrane bound enzymes (phosphodiesterase and 5'-nucleotidase) are capable of degrading extracellular cAMP to adenosine (343), which then act in an autocrine/paracrine fashion to inhibit adenylate cyclase activity. This may represent a transmembrane negative feedback mechanism, which would limit excess intracellular cAMP production, and inhibit lipolysis (342).

The major prostaglandins (PGs) released from both rodent and human adipose tissue are prostacyclin (PGI_2) and PGE_2 (344). PGE_2 has been shown to be a potent anti-lipolytic agent (345) while PGI_2 stimulates lipolysis in rat adipocytes (345).

3. Lipolysis in different fat depots and in normal and obese subjects

There are substantial evidence that lipolysis in different fat depot is different. This is true in both rodents and humans. In rats, sensitivity of subcutaneous adipocytes to β -adrenergic receptor agonists is lower than that of internal adipocytes (346). This is associated with the decreased expression of β_1 , β_2 , β_3 -receptors and increased expression of α_2 -receptors in subcutaneous adipocytes compared to internal adipocytes (346). In addition, HSL activity, protein levels and mRNA levels are also lower in subcutaneous than in internal adipocytes (346,347).

However, depot differences in lipolysis rate in humans are more complex. In general, basal lipolysis rate is higher in upper body adipose tissue than in lower body adipose tissue; however, there is no gender difference (348,349). In addition, basal lipolysis correlates positively with fat cell size (350,351), so that larger adipocytes have greater basal lipolytic activity per cell. Release of FFA from lower-body adipose tissue increases more in response to epinephrine in men than in women, whereas palmitate release from upper-body fat increases in both men and women upon epinephrine stimulation (352,353). This suggests that catecholamine-mediated lipolysis may play a role in determining gender based differences in body fat distribution. Further, lipolysis is low in peripheral subcutaneous adipose tissue, intermediate in the subcutaneous abdominal site, and high in the visceral adipose regions (354,355). This can be attributed to regional variations in the function of insulin receptors and adrenoceptors of fat cells (354,355).

Recent data demonstrated that abdominal subcutaneous adipocytes taken from different individuals exhibit variations in the lipolytic rate in response to catecholamines, an adenylate cyclase activator forskolin, and a cAMP analog dibutyryl cAMP (356). The variations in lipolytic rate are correlated to HSL activity, protein content and mRNA levels (356). This suggests that in addition to short-term regulation of HSL, lipolysis is also regulated by the amount of HSL available in adipocytes (356).

Lipolytic rate is also altered between obese and lean subjects. Several studies have shown that after an overnight fast the turnover rates of free fatty acids and glycerol in vivo as well as the basal release of glycerol from fat cells in vitro are increased in obese subjects (357-359). This is caused in part by a combination of increased fat cell size and

increased total mass of adipose tissue. However, resistance to catecholamine-stimulation of lipolysis has been frequently observed in obese subjects (360,361), which may contribute to the excessive weight gain during caloric excess and to insufficient weight loss during caloric deprivation. This is associated with a decrease in β -adrenergic receptor expression (362) or an increase in α 2-adrenergic receptor numbers (363). Multiple defects in catecholamine-induced lipolysis are also observed in the fat cells in cases of human obesity (364). There are controversial results regarding adipose tissue resistance to insulin's anti-lipolytic effect in obesity. However, greater resistance to the anti-lipolytic effect of insulin and to the lipolytic effect of catecholamines is found in upper-body obesity as compared with lower-body obesity (359,365), and the basal rate of free fatty acid release was higher in upper-body obese subjects than in normal or lower-body obese subjects (359,365). This is consistent with a stronger association between upper-body obesity and the metabolic and cardiovascular complications of overweight (366). Recent population studies revealed that a polymorphic marker at the HSL gene is associated with abdominal (upper body) obesity and type II diabetes (367), suggesting a putative role of HSL in the pathogenesis of type II diabetes and abdominal obesity (367).

V. REGULATION OF PANCREATIC β -CELL FUNCTION IN NORMAL AND OBESITY/DIABETES

A. Regulation of insulin release in pancreatic β -cells

The polypeptide hormone insulin is synthesized by pancreatic β -cells and plays an important role in glucose homeostasis. Insulin secretion is regulated by both nutrients and neurotransmitters. Glucose is the predominant nutrient secretagogue, while several other nutrients, including some amino acids, ketoacids, ketone bodies and fatty acids also influence insulin release (368-370). This makes the β -cell a “fuel sensor” which integrates the signals of nutrients and modulators to secrete insulin according to the needs of the organism (368-370). Alterations of pancreatic β -cell function result in metabolic disorders such as obesity and diabetes (371).

1. Nutritional regulation of insulin secretion

Glucose stimulated insulin secretion (GSIS) requires glucose metabolism by β -cells. Insulin release can be stimulated by glucose and other metabolizable sugars such as mannose, but not by poorly metabolized sugars galactose, xylose or L-arabinose (372). In addition, non-metabolizable sugar analogs such as 2-deoxyglucose or 3-O-methyl glucose are ineffective in stimulating insulin release (372), while inhibitors of glycolysis (mannoheptulose or glucosamine) at the level of glucose phosphorylation blocks glucose stimulation of insulin release (373).

Glucose enters the β -cell via the glucose transporter Glut-2 (374). Glucose transport may not be a regulatory or rate-limiting step in GSIS, as islets are capable of rapid equilibration of glucose between the interior of the β -cells and the plasma (368,369).

Glut-2 has been found to be downregulated in animal models of diabetes, including db/db, Zucker diabetic fatty rats, streptozotocin-injected neonatal rats, and rats rendered diabetic by dexamethasone injection (375). In addition, a point mutation in the Glut-2 gene has been identified in a single NIDDM patient (376). Further, overexpression of both Glut-2 and glucokinase restored glucose responsiveness in rat insulinoma cells (RIN) (369). These observations suggest that Glut-2 is necessary but not sufficient in GSIS, as normal GSIS also requires β -cell glucokinase, and possibly other factors as well (369).

Glucose is phosphorylated by members of the hexokinase family, which includes hexokinase I, II, III and IV. The hexokinase IV is also called glucokinase (GK). Hexokinase I, II and III are distinguished from GK by a lower K_m for glucose (10-100 μ M vs. approximately 8 mM), and allosteric inhibition by glucose-6-phosphate (369). Islet homogenates contain both high K_m and low K_m glucose kinase activity (377), which is attributed to glucokinase (GK) and multiple hexokinases, respectively (377,378). Evidence indicates that GK controls glycolytic flux at physiological glucose concentrations and serves as a "glucose sensor" (368,369). Consistent with this, mutations in the GK gene have been identified in patients with maturity-onset diabetes of the young (MODY), a rare form of type 2 diabetes (379), resulting in higher glucose concentration threshold in triggering insulin release (380). In contrast, alterations in the expression of the low K_m hexokinases may be implicated in increased insulin secretion at basal glucose levels in some obese and diabetic individuals and obese Zucker rats (381). Variations in the expression of low K_m hexokinases also explain alterations in glucose responsiveness in β -cell sub-populations (382).

It is well accepted that GSIS is triggered by glucose metabolism induced alterations in membrane potential and Ca^{2+} influx. Following a rise in glucose, the rapid phosphorylation of glucose by GK generates ADP, resulting in a transient decrease in ATP/ADP ratio. This leads to an increase in glycolysis via phosphofructokinase (PFK) activation and stimulation of mitochondria activity via the activation of mitochondria dehydrogenases and the respiratory chain. This results in increased production of ATP. The resulting increase in ATP/ADP ratio cause the closure of K^+_{ATP} channel and subsequent opening of voltage-dependent Ca^{2+} channels. The resulting accelerated Ca^{2+} influx via L-type voltage dependent Ca^{2+} channels initiates exocytosis, which is due to Ca^{2+} activation of protein kinases that interact with components of the microtubular/exocytotic machinery (369,383).

This ionic hypothesis of insulin secretion indicates a critical role for K^+_{ATP} channel and Ca^{2+} influx in GSIS. Cloning of β -cell K^+_{ATP} channels and studies thereafter demonstrate that the β -cell K^+_{ATP} channels are comprised of two types of subunits: Kir6.2, a member of the inwardly rectifying K^+ channel family, and SUR, the high affinity sulphonylurea receptor, a member of the ATP-binding cassette-traffic ATPase superfamily (384). Selective inhibition of K^+_{ATP} channels with sulphonylureas (glibenclamide, tolbutamide etc.) induces insulin release (383). Conversely, K^+_{ATP} channel openers, such as diazoxide, inhibit insulin secretion (383). In addition, mutations at the SUR gene lead to a loss of β -cell K^+_{ATP} channel activity, and cause an autosomal recessive disorder, persistent hyperinsulinemic hypoglycemia of infancy (PHHI), which is characterised of excessive insulin secretion despite profound hypoglycemia (384). On the other hand, stimulating Ca^{2+} influx with a depolarization agent, KCl, induces insulin

release, while depletion of extracellular Ca^{2+} or addition of L-type Ca^{2+} channel antagonists such as nifedipine inhibits glucose stimulated insulin release (383, 385).

The FAD-linked mitochondrial glycerol phosphate dehydrogenase (FAD-GPDH), the component of the glycerol phosphate shuttle which transfers reducing equivalents produced in glycolysis (NADH) to the mitochondria, is about 50-fold higher in the islets than in other tissues (386), and is markedly activated by Ca^{2+} (387,388). Increased FAD-GPDH may serve to accelerate the transfer of reducing equivalents to the mitochondria during β -cell activation by glucose, and promote rapid reoxidation of NADH to maintain high glycolytic flux and production of ATP (368). Interestingly, pancreatic β -cells contain very low lactate dehydrogenase (LDH) activity (389), which suggests an important role for mitochondria glycerol phosphate shuttle in the regeneration of NAD^+ . In addition, deficiency in FAD-GPDH activity has been found in the islets of GK rats, a rodent animal model of type 2 diabetes (390).

Inhibition of mitochondrial pyruvate transport by α -cyano-4-hydroxy-cinnamate (α -CHC) greatly reduces GSIS (391). In addition, inhibitors of electron transport or uncouplers of mitochondrial ATP production, such as antimycin A, rotenone, cyanide and FCCP, block GSIS (392). These observations indicate that glycolysis flux and activation of mitochondria activity are important for GSIS. Consistent with this, nutrient secretagogues such as glucose, mannose, the triose glyceraldehyde-3 phosphate and dihydroxyacetone phosphate, and mitochondrial fuel methyl esters of succinate and 2-ketoisocaproate (KIC), the deamination product of leucine are also capable of increasing ATP/ADP ratio, the ratio of reduced purine nucleotide $[\text{NAD(P)H}]$ to oxidized purine nucleotide $[\text{NAD(P)}^+]$, and increase $[\text{Ca}^{2+}]_i$ (368,369,392,393).

Recent studies indicated that anaplerosis, the process of replenishing the citric acid cycle with metabolic intermediates, plays an important role in the stimulation of insulin release (368). Anaplerosis serves to accelerate the generation of ATP and, to cause an elevation in citrate, the precursor of malonyl CoA, the candidate metabolic coupling factor in β -cells (368,369). Indeed, glucose stimulation causes a rapid elevation of both citrate (394) and malonyl-CoA (395). In addition, “anaplerotic secretagogues” such as glutamine, dimethyl-succinate or dimethyl-fumarate potentiate leucine-induced insulin secretion (396,397). Pyruvate can be decarboxylated to acetyl-CoA via pyruvate dehydrogenase (PDH) or carboxylated to the citric cycle intermediate oxaloacetate (OAA) by pyruvate carboxylase (PC). PC activity is usually high in tissues with gluconeogenic or lipogenic activity. However, pancreatic islets express high pyruvate carboxylase (PC) activity, although it is not a gluconeogenic tissue and has very low level of lipogenesis (398,399). MacDonald et al reported that about 50 % of pyruvate are metabolized to citric acid cycle intermediates (400). In addition, acetyl-CoA carboxylase (ACC), which catalyzes the formation of malonyl-CoA and usually is abundant in lipogenic tissues, is also abundant in β -cells (395).

Recently, malonyl-CoA has been implicated in the stimulation of insulin release. Corkey et al reported that in response to glucose, β -cells exhibit a rapid rise in malonyl CoA content that precedes insulin release (395). Simultaneous suppression of palmitate oxidation and increased formation of DAG and other esterified products are also observed (395). Fatty acid oxidation is the major energy source in resting β -cells (401). It is hypothesized that glucose metabolism results in increases in malonyl CoA levels, which in turn inhibits mitochondrial carnitine palmitoyl-transferase I (CPT I). This results

in the inhibition of fatty acid oxidation, leading to a shift from fatty acids to glucose as oxidative fuel, and an elevation of cytosolic long-chain fatty acyl-CoAs (FA-CoA) (368,369). These long-chain FA-CoAs are proposed to act as proximal signals for insulin release (402). This model has been substantiated in cell cultures in which hormone release is associated with increased production of malonyl-CoA (395,403). This was associated with a 15-fold increase in acetyl-CoA carboxylase (ACC) mRNA levels, which catalyzes the formation of malonyl-CoA from acetyl-CoA (403). Inhibition of CPTI with 2-bromopalmitate or etomoxir enhanced glucose-induced insulin secretion, as does exogenous FFA (402,404). In addition, (-)-hydroxycitrate (HC), an inhibitor of APT-citrate lyase that catalyzes steps leading to the formation of malonyl-CoA, markedly inhibited insulin secretion stimulated by glucose (404).

Long-chain FA-CoA may directly or indirectly be involved in insulin release. They may activate protein kinase C via the formation of diacylglycerol (405). They have also been found as regulators for K_{ATP} and/or Ca^{2+} channel activities (406), and may be involved in the acylation of key proteins in exocytosis (401).

Recent evidence indicates a K^+_{ATP} channel independent mechanism in GSIS (407,408). Gembal et al reported that opening K^+_{ATP} channels by diazoxide blocked the effect of glucose on mouse pancreatic β -cell insulin release but did not prevent glucose potentiation of 30 mM KCl-induced insulin release. This effect was not accompanied by changes in β -cell membrane potential, ^{45}Ca flux, or $[Ca^{2+}]_i$ (407,408). However, it did require an initial elevation of $[Ca^{2+}]_i$. This effect was not ascribed to phosphoinositide metabolism or activation of protein kinase A or C. In stead, it requires glucose metabolism (407,408). Indeed, the increase in long-chain FA-CoA and their derivatives

in response to glucose may in turn serve as distal signal and modulate K_{ATP} and Ca^{2+} channel activities (368,369). Increase in $[Ca^{2+}]_i$ may exert its effect on exocytosis, along with bioactive phospholipids such as diacylglycerol (DAG), via the activation of protein kinases that are implicated in the exocytotic process (369).

2. Neuroendocrine regulation of insulin secretion

Insulin secretion is also regulated by peptide hormones and neurotransmitters. Acetylcholine and catecholamines are important neurotransmitters that promote and inhibit, respectively, insulin release (409, 410). The hormone somatostatin derived from islet δ -cells exerts potent inhibitory effect while glucagon, derived from islet α -cells, stimulates insulin release (409,410). Hormones derived from gut, namely “incretins”, such as glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP) potently promote glucose-induced insulin release (409,411). The incretins form the “enteroinsular axis” and are important regulators of carbohydrate metabolism (411).

Non-glucose secretagogues, which usually only modulate the secretory response, bind to specific membrane receptors and activate various second messenger systems, which in turn alter protein phosphorylation and phospholipid metabolism, leading to changes in $[Ca^{2+}]_i$ levels and trigger insulin secretion (409).

cAMP is elevated by hormones such as glucagon, GLP-1 and GIP in β -cells (409). The increase in cAMP directly induces an increase of $[Ca^{2+}]_i$ through activation of L-type Ca^{2+} channels. Forskolin, an adenylate cyclase activator, stimulated ^{45}Ca uptake and electric activity in pancreatic islets in the presence of 10 mM glucose (412). This effect was blocked by omission of extracellular Ca^{2+} and Ca^{2+} -channel blockers (412). Forskolin also augmented the effect of tolbutamide, a potent sulfonylureas on $[Ca^{2+}]_i$

(413). In addition, the potentiation of insulin release by cyclic AMP involves sensitization of the secretory machinery to Ca^{2+} (409). The increase in cAMP by forskolin or isobutyl methylxanthine (IBMX), a phosphodiesterase inhibitor, augmented insulin release in permeabilized rat pancreatic islets in which intracellular Ca^{2+} concentrations were held constant by EGTA infusion (414). It is suggested that cAMP may directly interact with the secretory machinery by increases in phosphorylation of protein kinase A substrates in β -cells (409). However, the actual mechanisms remain unidentified.

Hormones and neurotransmitters such as acetylcholine and arginine vasopressin (AVP) stimulates phospholipase C activity, leading to an increase of inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) in the presence of glucose (409, 415). This results in mobilization of Ca^{2+} from IP_3 -sensitive stores in the endoplasmic reticulum and an increase in $[\text{Ca}^{2+}]_i$, which triggers insulin release (409).

DAG is a potent activator of protein kinase C (PKC), which plays an important role in modulating insulin secretion (409). The effect of the muscarinic agonist carbachol on glucose induced insulin release is exerted through the accumulation of DAG and subsequent activation of PKC (409). In addition, stimulation of PKC by the phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA) in mouse islets amplified the effect of tolbutamide on insulin release without affecting its ionic and electrical effects (416).

In addition, significant cross-talk between cAMP and PLC signaling pathways has been demonstrated. In HIT cells, forskolin markedly increases the frequency, amplitude and duration of Ca^{2+} transients evoked by hormones linked to PLC, such as AVP (417). Forskolin also potentiates AVP-induced insulin secretion from HIT cells in the presence

of 10 mM glucose (417). This indicates that synergistic cross-signaling between the cAMP- and Ca^{2+} -phosphoinositide signaling pathway may be important for the regulation of insulin release.

In contrast, norepinephrine (α_2 -adrenergic agonist), somatostatin and galanin serve to inhibit insulin release by coupling to pertussis toxin-sensitive G_i and G_o proteins, resulting in the inhibition of adenylate cyclase and the decrease in cAMP levels (411). In addition, they also exert their inhibitory action via the activation of K_{ATP} channel, inhibition of Ca^{2+} channel and inhibition of exocytosis (411).

3. Effects of melanocortin peptides on insulin release

Proopiomelanocortin (POMC) and ACTH-like peptides have been shown to be produced by rat islets (418), and the secretion of ACTH-like peptides is increased along with insulin in response to increased concentrations of glucose in culture medium (419). In addition, picomolar concentrations of $1\text{-}^{39}\text{ACTH}$ have been shown to stimulate insulin release in the presence of either 3.3 or 16.7 mM glucose; the stimulatory effect is more profound at 16.7 mM glucose (419,420). The effect of ACTH is associated with inhibition of membrane ATPase activity, resulting in an increase in $[\text{Ca}^{2+}]_i$ (420). However, ACTH does not affect membrane potential (420). Several melanocortin receptors have been found in pancreas (82,89). However, the physiological significance of ACTH stimulation of insulin release is unclear.

4. Effect of leptin on insulin release

The functional long-form leptin receptor has been shown to be expressed in pancreatic islets (421). In addition, leptin exerts potent inhibitory effect on insulin release (421,422,424). This is exerted via the ability of leptin to activate K_{ATP} channel in β -cells

(422,423), resulting in hyperpolarization. Leptin is also shown to lower $[Ca^{2+}]_i$ in β -cells (424). It is suggested that leptin resistance in obesity may cause chronic hypersecretion of insulin, which may result in hyperinsulinemia, and further promote obesity (424).

B. Amylin: regulation of its release and physiological functions

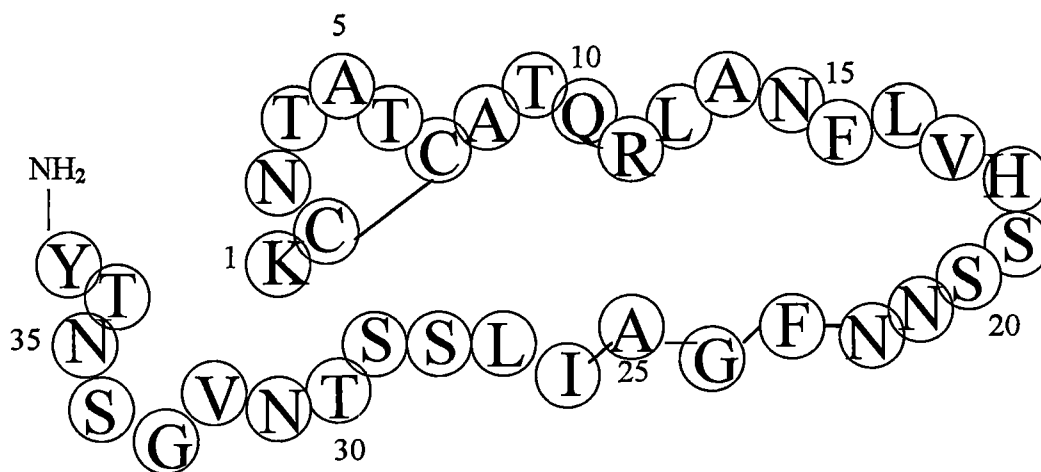
Islet amyloid deposits were first described in the pancreas of patients with type 2 diabetes at the beginning of this century (425). They consist of aggregates of small proteins forming fibrils of 7-10 nm with β -pleated sheet structures (426), which line up between β -cells and endothelial cells and islet capillaries (427,428). However, The major component, islet amyloid polypeptide (amylin), was not identified until 1987 (429).

Amylin is a 37-amino acid polypeptide primarily produced by pancreatic β -cells (Figure 6A). It is a member of the calcitonin (CT)/calcitonin-gene-related peptide (CGRP) family, which includes CT, CGRP, amylin and adrenomedullin (430). Amylin bears a 46 % homology with human CGRP and 15 % with human CT (430). All four peptides contain two N-terminal cysteines forming a disulfide bond that results in an N-terminal loop, and a C-terminal amide, which are essential for their full biological activity (430).

1. Regulation of amylin release

Amylin is co-localized with insulin in β -cell secretory granules (45). In addition, amylin is co-secreted with insulin in response to glucose and non-glucose secretagogues (46,431,432). In both rat islets and HIT-T15, a hamster pancreatic β -cell line, both amylin and insulin are stimulated in a parallel fashion by glucose (46,431) and the non-glucose secretagogues arginine (46,431), leucine plus glutamine (431), and potentiation of glucose's effect by theophylline, a phosphodiesterase inhibitor (431). Both hormones

A



B

Species	Amino acids										Amyloidogenesis
	20					25				29	
Human	S	N	N	F	G	A	I	L	S	S	+++
Monkey	--	--	--	--	--	T	--	--	--	--	+++
Cat	--	--	--	L	--	--	--	--	--	--	+++
Rat	--	--	--	L	--	P	V	--	P	P	--
Mouse	--	--	--	L	--	P	V	--	P	P	--
Hamster	N	--	--	L	--	P	V	--	S	P	--

Figure 6. Amino acid sequence of amylin of different species. A. The amino acid sequence of human amylin, which contains a C-terminal amide and a N-terminal disulfide bond. B. Comparison of the amino acid sequences from residues 20 to 29 in the amylin peptide among different species. Line represents identical amino acids. Substituting proline at position 25, 28 and 29 in rodent amylin greatly reduces the tendency of amyloidogenesis in these species.

are also similarly stimulated by sulfonylureas glipizide (431), tolbutamide and glyburide (432), cholinergic stimulation by carbamylcholine (431) and peptide hormones vasoactive intestinal peptide (VIP) (431) and glucagon (46,431). The molar ratio of amylin to insulin remains 1 % for rat islets (431,432) and 0.4 % for HIT cells (46). In addition, both amylin and insulin secretion are similarly inhibited by an inhibitor of glycolysis, mannoheptulose, depletion of extracellular calcium, activation of K_{ATP} channel by diazoxide, α_2 -adrenoreceptor agonist epinephrine, islet hormone somatostatin, a β -cytotoxin (streptozotocin) and a cytokine (interleukin- 1β) (431). These studies indicate that amylin and insulin secretion is regulated via similar mechanisms. In addition, German et al reported that the insulin and amylin genes contain similar promoter elements that bind identical β -cell nuclear complexes (433), and glucose similarly regulates insulin and amylin expression in rat pancreatic islets (434).

Oral or intravenous glucose loading and meal ingestion results in similar increase in blood insulin and amylin levels in healthy subjects (435-439), with changes in insulin levels correlates with changes in amylin levels (438). Basal amylin levels in normal subjects range from 4 to 8 pM (435,436,438,439), which increases 2-6 fold after glucose or meal loading, reaching peak concentrations of 15-25 pM (435-439). Basal and stimulated amylin levels are higher in obese patients with either normal or impaired glucose tolerance (NGT and IGT, respectively), and in obese patients with diet-controlled NIDDM (438,439). In non-obese patients with IGT and diet-controlled NIDDM, basal and stimulated amylin levels is either not different or slightly increased compared to healthy subjects (438). However, both basal and stimulated amylin levels are significantly lower in insulin-dependent diabetes mellitus (IDDM) patients and patients

with uncontrolled NIDDM (437-439). These changes parallel with changes in blood insulin levels (437-439). In addition, plasma amylin levels are elevated in obese/diabetic animal models such as *ob/ob*, *db/db* and *A^{vy}* mice (440,38)

However, dissociated secretion of amylin and insulin has also been observed. Increased amylin-to-insulin molar ratio in response to glucose stimulation has been observed in rats rendered hyperglycemic by streptozotocin (441), in Zucker diabetic fatty rats (442) and in humans with IGT (437). These observations suggest that amylin and insulin secretion or synthesis may be independently regulated under some conditions.

2. Potential physiological function of amylin

Numerous studies have been performed to investigate the physiological function of amylin. Despite these efforts, the function of amylin on glucose metabolism remains conflicting. Some studies demonstrated that amylin inhibits peripheral glucose disposal by inhibiting muscle glycogen synthesis (443), increasing muscle glycogenolysis (444), and inhibiting glycogen synthesis in hepatocytes (445). Amylin injection also causes elevation of plasma lactate, glucose and reduced insulin sensitivity in rats (446,447). However, most of these studies utilize pharmacological concentrations of amylin, and these effects have not been confirmed at more physiological concentrations. In addition, most studies are performed in rodents, and no studies in humans have been able to confirm an amylin-mediated reduction in insulin sensitivity (448-450).

Recently, Young et al reported that gastric emptying is accelerated in diabetic BB rats, an IDDM animal model lacking both insulin and amylin (47). Amylin injection dose-dependently delayed the rise in plasma glucose levels after oral glucose load by slowing the rate of gastric emptying, with an ED₅₀ of 20 pM (47). Similar effects have

been observed by others (451-453). This effect is more potent than glucagon-like polypeptide-1 (GLP-1) and cholecystokinin (CCK) (452). These data suggest that amylin may serve to delay glucose input into the blood, thereby coordinating with insulin in modulating blood glucose levels under physiological conditions. Consistent with this, the human amylin analogue pramlintide, in which amino acid residues at position 25,28 and 29 have been substituted with proline (454), has been shown to exert the same effect as amylin, slowing the rate of gastric emptying in type 1 diabetes patients (455,456). Pramlintide also improves postprandial (457) and long-term (28 days) glycemic control in both type 1 and type 2 diabetes patients (458,459).

Amylin has been shown to reduce food intake in rats and mice after peripheral and central administration (460-465). The anorectic effect of amylin occurs at close to physiological concentrations (464,465). Amylin immunoreactivity and binding sites have been detected in brain regions (430,466). Of the particular interest is the area postrema/nucleus of the solitary tract (AP/NTS) region of brain stem, which not only receive peripheral satiety signals via splanchnic and vagal afferents, but is also exposed to blood borne signals due to the lack of blood brain barrier in this region. Destruction of this region significantly reduced the anorectic effect of amylin (467,468). In addition, the area postrema, along with dorsal motor nucleus of the vagus, is part of a neural loop implicated in the control of gastric motility. It is hypothesized that the effect of amylin on gastric emptying is also mediated through the area postrema (469).

There are also conflicting results in the effect of amylin on insulin secretion. Some studies found that amylin suppresses insulin secretion (470), while other studies can not confirm this effect (471). Most of these studies are done in rodents. Bretherton et al

reported that high concentrations (100 times postprandial levels) is required to suppress insulin secretion to an intravenous glucose load in humans (472). More recently, inhibition of endogenous amylin activity with either an amylin antiserum or an amylin antagonist (amylin-(8-37)), dose-dependently increased arginine- and glucose-stimulated insulin release in rat islets (473). Arginine-stimulated glucagon and somatostatin secretion was also dose dependently increased by amylin antiserum and antagonist treatment (473). As intraislet amylin concentration may reach substantially higher levels than that found in peripheral circulation, these observations suggest that intraislet amylin may tonally inhibit insulin, glucagon and somatostatin secretion via paracrine actions (473). In the same study with rat islets, the authors also demonstrate that insulin and glucagon secretion in response to arginine is also increased by somatostatin antiserum treatment (473). This may serve as an intraislet feedback control to prevent oversecretion of hormonal products from islet cells (473). This is not surprising in light of evidence that somatostatin inhibits insulin, glucagon and amylin secretion (431,473), and that glucagon stimulates both insulin and amylin secretion (45,431), which indicates an extensive inter-regulatory loop among islet hormones.

C. Pancreatic islet amyloid deposits: role in the development of type 2 diabetes

Islet amyloid is an amorphous mass of fibrils composed of stacked sheets of amylin in a β -pleated conformation held together by hydrogen bonding (428, 474). This structure confers a considerable degree of insolubility to the amyloid deposits (474). It usually accumulates in extracellular spaces and juxtaposes to β -cell membranes (428,429,474) and is a characteristic of type 2 diabetes (427,428). In pronounced islet amyloidosis, a majority of islets are almost completely converted to amyloid (474). Islet amyloid

deposits are found in humans, monkeys, cats and raccoons, but not in rats, mice, dogs and guinea pigs (427,428). The amyloidogenicity of amylin is chiefly linked to the 20-29 region of amylin. The presence of proline residues in positions 25, 28 and 29 in amylin species of rats and mice inhibits the formation of β -sheets (475), a necessary step for amyloid generation, thereby preventing amyloid formation (476) (Figure 6B).

Islet amyloid deposition progresses with disease, and is accompanied by a significant decrease of β -cell mass (477,478). In addition, human amylin appears to be toxic to β -cells (479). This toxicity is mediated by the fibrillar form of the amylin peptide and requires direct contact of the fibrils with the cell surface. Cat amylin, which is also amyloidogenic, also induces islet cell death, while the non-amyloidogenic rat amylin is not toxic to islet β -cells (479). Islet cell toxicity can be first detected at 5 μ M of human amylin, and complete cell death occurs at 20 μ M amylin. The mechanism of cell death involves RNA and protein synthesis and is characterized by plasma membrane blebbing, chromatin condensation and DNA fragmentation, indicating that amylin induces islet cell apoptosis (479).

The mechanism of amyloid fibril formation is not known. Although humans express an amyloidogenic species of amylin, amyloid deposits rarely occurs in non-diabetic individuals (427). In addition, most lines of transgenic mice overexpressing human amylin in β -cells do not spontaneously develop islet amyloid deposits and hyperglycemia (480-483). One strain of homozygous transgenic mice overexpressing human amylin did develop hyperglycaemia (484). However, this is associated with small intra- and extracellular amorphous amylin aggregates resulting in cytotoxicity and β -cell death, instead of large extracellular amyloid deposits that are commonly present in type 2

diabetic patients (484). In addition, two recently developed immortal mouse pancreatic β -cell lines overexpressing human amylin also do not spontaneously develop islet amyloid (485). On the other hand, introducing the *A^{vy}* trait, which is characterized by obesity and insulin resistance, into these transgenic animals did result in extensive islet amyloid formation, leading to reduced β -cell mass and type 2 diabetes (49). Similarly, feeding these transgenic mice a high-fat diet, or introducing the *ob* trait into these animals, both of which induce obesity and insulin resistance, both resulted in accelerated amyloidogenesis and profound hyperglycemia (486,487).

These data suggest that production of an amyloidogenic amylin by itself is necessary but not sufficient to induce amyloid formation, whereas the interaction between obesity/insulin resistance status and amylin expression appears to be important in islet amyloidogenesis. Islet amyloid formation may be both a consequence of obesity/insulin resistance and a cause of β -cell failure and subsequent hyperglycemia in the later stage of type II diabetes.

Amylin is not the sole components of amyloid deposits. Examination of type 2 diabetic patients has demonstrated the presence of at least two additional components in islet amyloid: apoE and the heparan sulfate proteoglycan perlecan (488). These proteins have also been identified in other forms of amyloid, such as Alzheimer's disease (489). ApoE is involved in lipoprotein metabolism. The human apoE gene is polymorphic, resulting in the expression of three different isoforms in humans (488). Homozygosity for the isoform apoE2 is associated with an increase in plasma intermediate-density lipoproteins and the development of premature atherosclerosis (490). Human islets normally do not express apoE (488). However, it is present in islet amyloid deposits, and

appears to be the only apolipoprotein present in islet amyloid deposits (488). This suggests that it may be transported to pancreatic islets via the circulation (488). The potential role of apoE in islet amyloidogenesis is not fully understood. However, transgenic mice overexpressing human amylin only develop islet amyloid deposits when fed with a high-fat diet (486) or introducing *A^{vy}* (49) or *ob* trait (487), all of which results in obesity, insulin resistance and dysregulated lipid metabolism. Considering the role of apoE in lipoprotein metabolism, it is possible that the development of islet amyloid deposits may be related to dysregulated lipid metabolism.

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PART 3

THE AGOUTI GENE PRODUCT STIMULATES PANCREATIC β -CELL CA^{2+} SIGNALING AND INSULIN RELEASE¹

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I. ABSTRACT

Ubiquitous expression of the mouse *agouti* gene results in obesity and hyperinsulinemia. Human *agouti* is expressed in adipose tissue, and we found recombinant agouti protein to stimulate lipogenesis in adipocytes in a Ca^{2+} -dependent fashion. However, adipocyte-specific *agouti* transgenic mice only became obese in the presence of hyperinsulinemia. Since intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) is a primary signal for insulin release, and we have shown agouti protein to increase $[\text{Ca}^{2+}]_i$ in several cell types, we examined the effects of agouti on $[\text{Ca}^{2+}]_i$ and insulin release. We demonstrated the expression of *agouti* in human pancreas, and generated recombinant agouti to study its effects on Ca^{2+} signaling and insulin release. Agouti (100 nM) stimulated Ca^{2+} influx, $[\text{Ca}^{2+}]_i$ increase and a marked stimulation of insulin release in two β -cell lines (RIN-5F and HIT-T15; $p < 0.05$). Agouti exerted comparable effects in isolated human pancreatic islets and β cells, with a 5-fold increase in Ca^{2+} influx ($p < 0.001$) and a 2.2-fold increase in insulin release ($p < 0.01$). These data suggest a potential role for agouti in the development of hyperinsulinemia in humans.

II. INTRODUCTION

Dominant mutations in the promoter region of the mouse *agouti* gene results in ectopic expression of this gene and an associated syndrome of obesity, hyperinsulinemia/insulin resistance and yellow coat color (1,2). The murine *agouti* gene is normally transiently expressed only in neonatal skin, where it regulates melanocyte pigment production. However, dominant mutations in the promoter region result in ubiquitous expression of normal agouti protein throughout life.

The mechanism of agouti modulation of coat color development is largely understood. This regulation occurs via competitive receptor antagonism of the α -melanocyte stimulating hormone (α -MSH) at its receptor (melanocortin 1 receptor; MC1-R), resulting in an inhibition of cAMP production and a consequent switch from eumelanin (black pigment) to phaeomelanin (yellow pigment) production (3,4). This competitive antagonism of melanocortin binding has served as a paradigm for the mechanism of agouti's action in obesity, and it has been proposed that competitive antagonism of another melanocortin receptor may be responsible for the metabolic effects of this gene (5). However, the actual mechanism of agouti action in obesity and insulin resistance has not yet been elucidated. Indeed, we have recently demonstrated that agouti also regulates cellular functions, such as cell Ca^{2+} signaling, independently of melanocortin receptor antagonism (6,7).

Intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) appears to play an important role in the metabolic derangements associated with obesity and insulin resistance (8-10). We have found that recombinant agouti protein causes a dose-dependent increase in $[\text{Ca}^{2+}]_i$ in a variety of cells (6,11), including both murine and human adipocytes. Agouti protein also stimulates

both the expression and activity of fatty acid synthase (FAS), a key enzyme in *de novo* lipogenesis, and thereby increases triglyceride accumulation in a Ca^{2+} -dependent manner (12). Moreover, this stimulation can be mimicked by stimulation of Ca^{2+} influx with KCl (13) and blocked by Ca^{2+} channel antagonism (14). Accordingly, since the human homologue of *agouti* is expressed in adipose tissue (15), the human agouti protein may similarly exert paracrine effects on adipocyte $[\text{Ca}^{2+}]_i$ and thereby stimulate *de novo* lipogenesis and promote obesity.

Alternatively, it has been proposed that central effects of agouti, mediated by MC-R antagonism, result in hyperphagia and obesity. Huszar et al (5) recently reported that targeted disruption of the murine melanocortin-4 receptor (MC4-R), which is expressed in brain, resulted in a syndrome of hyperphagia and hyperinsulinemia similar to that found in dominant *agouti* mutations. This suggested that chronic antagonism of the central nervous system MC4-R may be a primary cause of agouti-induced obesity (5). However, although MC4-R expression is considered to be primarily limited to most regions of the brain, MC4-R mRNA is also expressed in muscle and adipose tissue (16,17). Consequently, the contribution of peripheral MC4-R antagonism to this hyperphagia/hyperinsulinemia syndrome is not clear.

We have recently reported that transgenic mice expressing *agouti* in adipose tissue under the control of the aP2 promoter become obese if supplemental insulin is concomitantly provided, while supplemental insulin does not produce this effect in their non-transgenic littermates (18). Since hyperplasia of pancreatic β cells precedes the development of obesity in agouti mutant mice (19), it is possible that hyperinsulinemia is a direct effect of agouti action on the pancreas and that this hyperinsulinemia, combined

with adipocyte *agouti* expression, may be responsible for the obesity syndrome found in *agouti* mutants. In support of this concept, we have noted that the effects of insulin and *agouti* on FAS expression are additive, and that the FAS promoter has an *agouti* response region distinct from its insulin-response element (20).

Accordingly, since we have previously shown *agouti* to regulate $[Ca^{2+}]_i$ in several cell types (6), and since increasing $[Ca^{2+}]_i$ is the proximate signal for insulin release (21), we have now evaluated the role of *agouti* in regulating pancreatic $[Ca^{2+}]_i$ and, consequently, insulin release. We report here that *agouti* is expressed and stimulates Ca^{2+} signaling in human pancreatic islets, as well as in β cell lines, thereby serving as a potent insulin secretagogue. This suggests a potential role for *agouti* in the development of hyperinsulinemia in humans.

III. MATERIALS AND METHODS

A. Isolation and purification of human pancreatic islets

Human pancreas was obtained from multiorgan donors and transported in cold University of Wisconsin (UW) solution (22-24). Islets were isolated within 6-8 hours by a modified method using collagenase digestion and Ficoll gradient centrifugation (22-24). Briefly, the pancreas was distended via the bile duct and perfused with collagenase type V (0.5 mg/ml) /type XI (2 mg/ml) dissolved in Hank's balanced salt solution and then digested in same solution at 37°C with shaking for 30 minutes. The tissue digest was then washed and sifted through a 500 µm nylon filter, and purified by Ficoll gradient centrifugation. Purified islets were maintained in primary culture in 6-well plates or 150 mm² culture dishes in RPMI-1640 medium supplied with 10 % FBS for 48 hours prior to experiment.

B. Cell culture

RIN-5F pancreatic β cell (ATCC, Rockville, MD) and primary human pancreatic islets were cultured in RPMI-1640 medium supplemented with 5 % fetal bovine serum (FBS; v/v) and antibiotics (50 units of penicillin/ml and 5 µg of streptomycin/ml). HIT-T15 pancreatic β cell (ATCC, Rockville, MD) was cultured in F-12K Nutrient Mixture supplemented with 10 % horse serum (v/v), 2.5 % FBS (v/v) and antibiotics. Cells were maintained at 37°C in an atmosphere containing 5 % CO₂ and 90 % humidity. Cell culture reagents (Horse serum, Trypsin-EDTA, antibiotics, Hank's balanced salt solution, DMEM, RPMI-1640, F-12K Nutrient Mixture) were obtained from Life Technologies (Grand Island, NY). FBS was obtained from Hyclone (Logan, Utah).

C. Production and purification of recombinant agouti polypeptide

A 614 bp XbaI/PstI fragment of the full-length mouse *agouti* cDNA (25) or human *agouti* cDNA (15) was subcloned into a baculovirus expression vector and expressed in *Trichophtisia ni* (*T.ni*) cells. Medium was collected 48 hours after infection and purified as previously described (26). The purified agouti protein was stored in PBS at -80°C prior to use. Previous data from our laboratory demonstrated that recombinant agouti protein dose-dependently increased $[Ca^{2+}]_i$ in several cell lines, including adipocytes, with an EC_{50} ranging from 18-62 nM depending upon cell type (23). Accordingly, a plateau dose of 100 nM agouti was used in the present study.

D. $[Ca^{2+}]_i$ determination

$[Ca^{2+}]_i$ values in RIN-5F, HIT-T15 pancreatic β cells and primary human pancreatic islets were determined fluorometrically as previously described (27,28). Briefly, cells were pre-incubated in serum-free medium for 2 hours, washed with Hank's balanced salt solution and trypsinized. Cells were collected by centrifugation, washed with HEPES-buffered salt solution (HBSS, containing components in mM: NaCl 138, $CaCl_2$ 1.8, $MgSO_4$ 0.8, NaH_2PO_4 0.9, $NaHCO_3$ 4, Glucose 0 or 5, referred to as glucose-free HBSS or HBSS, Glutamin 6, HEPES 20 and BSA 1 %). Cells were then loaded with fura-2 acetoxymethyl ester (10 μ M) in same solution for 20 minutes at 37°C in dark with continuous shaking, and $[Ca^{2+}]_i$ was measured using dual excitation (340 and 380 nm) and single emission (510 nm) fluorometry. Following the establishment of stable baseline (at approximately 100 seconds), the response to agouti (0-100 nM) was determined. Digitonin (25 μ M) and Tris/EGTA (both 100 mM, pH 8.7) were added at 500 and 550

seconds to determine maximal and minimal fluorescent ratios, respectively, and $[Ca^{2+}]_i$ was then calculated by the equation of Grynkiewicz et al (29).

E. Ca^{2+} influx measurement

Ca^{2+} influx was determined using the manganese (Mn^{2+}) quench technique. Mn^{2+} enters cells via Ca^{2+} -influx pathways and, once inside cells, quenches Ca^{2+} -fura-2 fluorescence (30-32). When measured at 360 nm, the isobestic wavelength of fura-2, the fall in fluorescence is indicative of Mn^{2+} influx irrespective of changes in $[Ca^{2+}]_i$ (30,32). Consequently, when excess Mn^{2+} is added to the medium, this Mn^{2+} quench technique serves as a surrogate measurement of Ca^{2+} -influx.

To measure Mn^{2+} influx in cells, HIT-T15 β cells or human pancreatic islets were loaded with fura-2 acetoxymethyl ester as described above for $[Ca^{2+}]_i$ determination. Fura-2 loaded cells were then washed with HBSS, and Mn^{2+} quench was measured fluorometrically by excitation at 360 nm and emission at 510 nm. After a stable baseline was established, $MnCl_2$ (0.2 mM for HIT-T15 cells and 1 mM for human pancreatic islets) was added at 50 seconds. Following the establishment of a new baseline (at 100 seconds), Mn^{2+} quench in response to agouti (0-100 nM) was determined. To study the effect of agouti on KCl-induced Ca^{2+} -influx, the Mn^{2+} -tracing was continued after the addition of agouti until 400 seconds and then KCl (5-50 mM) was added. Digitonin (50 μ M) was added at the end of experiment (at 500-550 seconds), allowing rapid Mn^{2+} -influx and quench of the fluorescence to basal level. The difference between this value and the initial value before the cells were exposed to Mn^{2+} was taken as 100 arbitrary fluorescence units (30,33). Mn^{2+} quench rate was estimated by linear regression of data collected after addition of each agonist or vehicle control (34).

F. Insulin release

Insulin release measurements were conducted by a modification of Shimizu et al (35). Briefly, RIN-5F and HIT-T15 cells were seeded in 6-well plates until confluent. Primary human pancreatic islets were maintained in 6-well plates for 48 hours after isolation. On the day of each experiment, cells were placed in glucose-free, serum-free medium for 2 hours and then incubated with either 100 nM agouti or the indicated test agents in the absence or presence of glucose for 2 hours. At the end of experiment, the medium was removed and stored at -80°C. Cells were sonicated in Homogenization Buffer (containing components in mM: sucrose 250, dithiothreitol 1, phenylmethylsulfonyl fluoride 0.1 and EDTA 1) and stored at -80°C for subsequent total protein correction. Immunoreactive insulin levels in the medium were measured by insulin Radioimmunoassay (RIA) Kits (Linco Inc. St.Charles, MO) for rat or human, as appropriate, and total protein was determined by modified Bradford method using Coomassie Blue dye (PIERCE, Rockford, IL)

G. RT-PCR and Southern blot analysis

Total RNA from human pancreas was isolated using CsCl₂ density centrifugation. mRNA from human pancreas were isolated according to manufacturer's instruction (Micro Poly (A) Pure Kit, Ambion Inc., Austin,TX). RT-PCR was conducted essentially as described by Kwon et al (15). Briefly, 500 ng of human pancreas mRNA was reverse-transcribed to first-strand cDNA using random-hexamer and reverse transcriptase (Perkin Elmer, Norwalk, CT) and amplified by PCR (Perkin Elmer, Norwalk, CT). The conditions of PCR were: 1 cycle (94°C 5 minutes), 35 cycles (94°C 30 seconds, 65°C 1 minutes, 72°C 1 minutes) and 1 cycle (72°C 7 minutes) with 0.5 μM 5' primer (5'-

ATGGATGTCACCCGCTTACTCCTGGCC-3') and 3' primer (5'-GCGCTCAGCAGTTGAGGCTGAGCACGC-3'), which corresponds to the 5' or 3' ends of the putative open reading frame of agouti gene (15). The amplified PCR products were then visualized by 1.2 % agarose gel electrophoresis, transferred to nylon membrane and hybridized with human *agouti* cDNA as a probe at 68°C overnight. The membrane was washed with 2XSSC/0.1 %SDS at room temperature for 30 minutes and then washed with 0.1XSSC/0.1% SDS at 65°C for 1 hour. The autoradiograph was exposed at -80°C for 30 minutes.

H. Statistics

All data are expressed as mean $\pm$ SEM. Statistical analysis of the data was performed by student t-test, paired student t-test or single factor analysis of variance (ANOVA) where appropriate.

IV.RESULTS

Recombinant murine agouti protein induced a gradual, sustained increase in $[Ca^{2+}]_i$ in hamster (HIT-T15) pancreatic β cells when incubated in glucose-free medium (Figure 7A), with 75 ± 14 nM increase over baseline (Figure 7B, $p < 0.05$ vs. control). In the presence of 5 mM glucose, the increase in $[Ca^{2+}]_i$ in response to 100 nM agouti was still significant, albeit somewhat decreased compared to that in the absence of glucose (Figure 7B). Agouti protein also stimulated a similar increase in $[Ca^{2+}]_i$ in rat (RIN-5F) pancreatic β cells (48 ± 2 nM increase over baseline, $p < 0.05$ vs. control).

100 nM agouti stimulated marked increase in insulin release in both cell lines in the absence of glucose, with a 1.4-fold increase in insulin release in HIT-T15 cell (54.24 ± 1.16 ng/mg protein vs. 77.30 ± 7.68 ng/mg protein, $p < 0.05$, Figure 8A), and a 2.2-fold increase in RIN-5F cells (6.78 ± 0.74 ng/mg protein vs. 14.98 ± 0.45 ng/mg protein, $p < 0.01$ vs. control).

To study the effect of agouti on glucose-stimulated insulin release, insulin release was determined in HIT-T15 cells incubated with agouti and different concentration of glucose following a period of glucose deprivation. As shown in Figure 8B, glucose stimulated insulin release from HIT-T15 cells in a dose-dependent manner, with half-maximal effect at approximately 2.5 mM and reached plateau at 5 mM (Figure 8B). The addition of 100 nM agouti enhanced glucose release from HIT cells both without glucose and with different concentrations of glucose (Figure 8B).

To investigate whether agouti modulation of Ca^{2+} is due to stimulation of Ca^{2+} -influx, Ca^{2+} -influx was monitored by Mn^{2+} -quench method in HIT-T15 cells. 100 nM agouti induced a 2-fold increase in Mn^{2+} -quench rate in these cells (Figure 9A, $p <$

Figure 7. Effect of the murine agouti protein (100 nM) on cytosolic Ca^{2+} levels ($[\text{Ca}^{2+}]_i$) in HIT-T15 pancreatic β cells. HIT-T15 cells were loaded with fura-2 acetoxymethyl ester (10 μM). $[\text{Ca}^{2+}]_i$ levels were measured using dual excitation (340 and 380 nm) and single emission (510 nm) fluorometry. During the Ca^{2+} -tracing of 600 seconds, 100 nM agouti was added at 100 seconds. Digitonin (25 μM) and Tris/EGTA (100 mM) were added at 500 and 550 seconds to determine maximal and minimal fluorescent ratios, respectively. $[\text{Ca}^{2+}]_i$ was then calculated by the equation of Grynkiewicz et al (16). A: upper panel: vehicle control; lower panel: agouti stimulation; B: Agouti-induced increase in $[\text{Ca}^{2+}]_i$ in the presence and absence of 5 mM glucose. Data are Mean $\pm$ SEM for 3-4 experiments. * $p < 0.05$ vs. Control

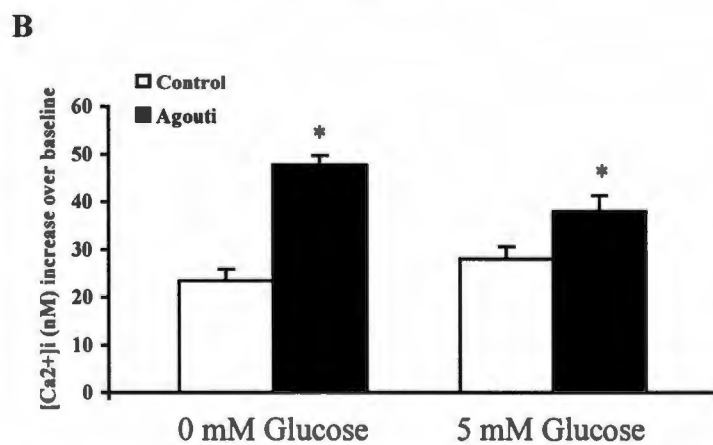
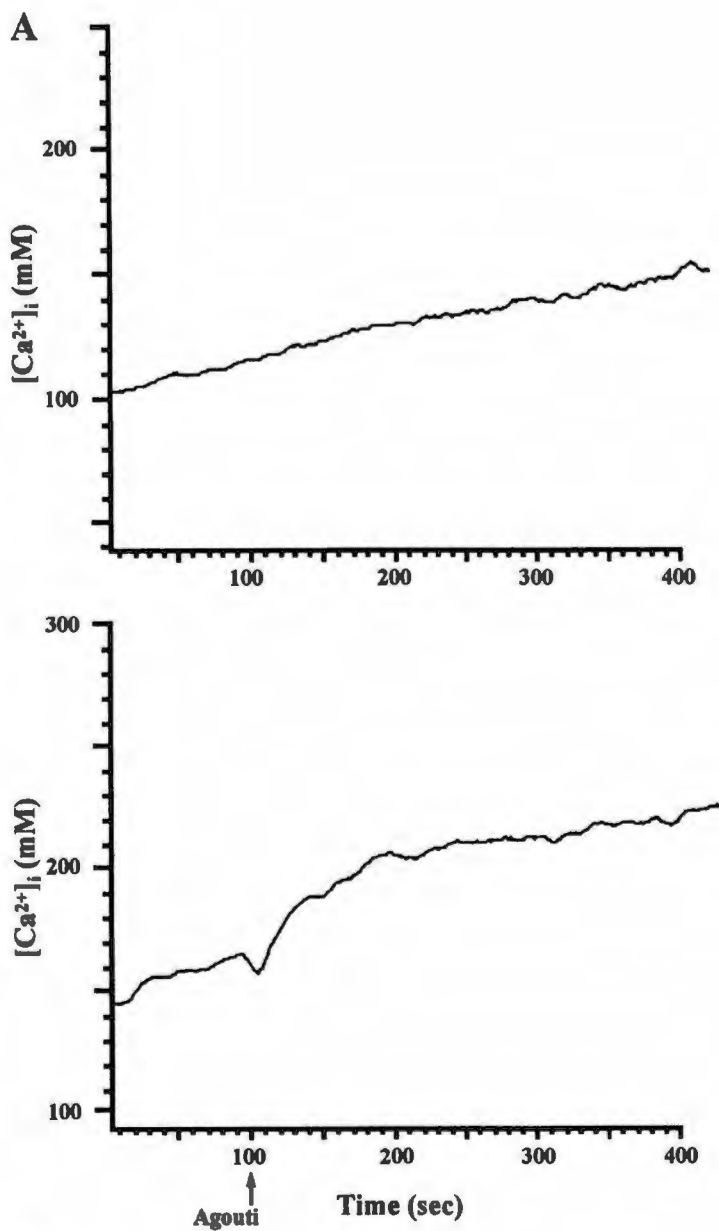
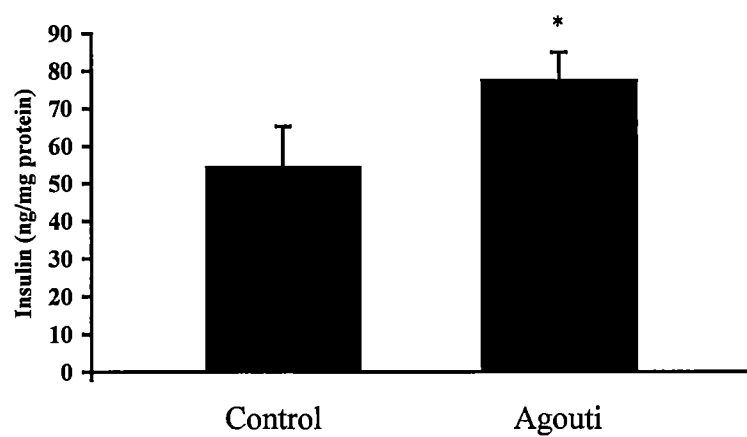


Figure 8. Effect of the murine agouti protein (100 nM) on insulin release in HIT-T15 in the absence of glucose (A) and in the presence of different concentrations of glucose (B). HIT-T15 cells were pretreated with glucose-free, serum-free medium for 2 hours and then incubated in the same medium with agouti (100 nM) and different concentrations of glucose for 2 hours. At the end of incubation, medium was removed and stored at -80°C prior to insulin measurement. Cells were sonicated and store at -80°C for subsequent protein correction. Data are the Mean $\pm$ SEM for 3-4 experiments. A: * $p < 0.05$ vs. Control. B: * $p < 0.05$ vs. Control. † $p < 0.05$ vs. previous glucose treatment. ‡ $p < 0.05$ vs. previous agouti treatment.

A



B

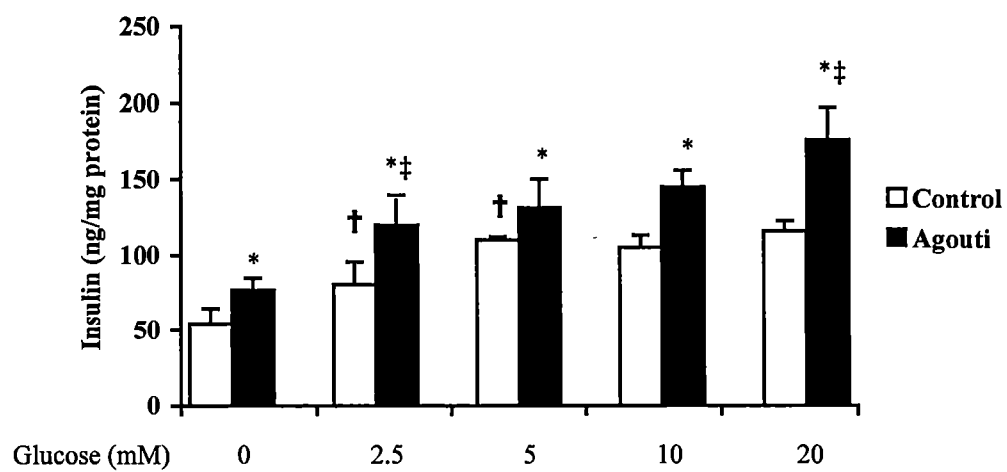
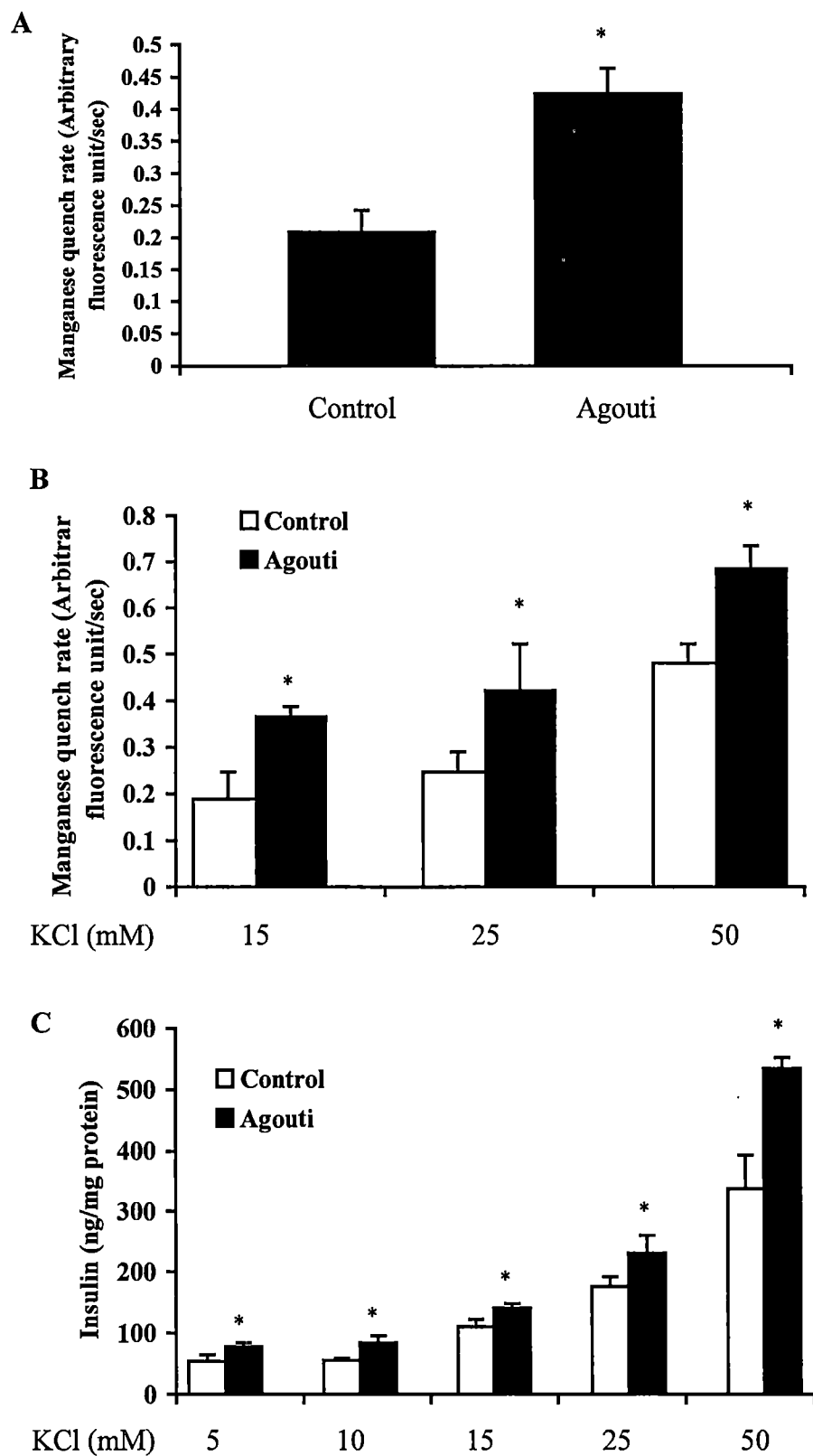


Figure 9. Effect of the murine agouti protein (100 nM) on Ca^{2+} -influx (A), KCl-induced Ca^{2+} -influx (B) and KCl-induced insulin release (C) in HIT-T15 pancreatic β cells in the absence of glucose. Ca^{2+} -influx is measured by the Mn^{2+} -quench technique with excitation at 360 nm and emission at 510 nm. HIT-T15 cells were loaded with fura-2 acetoxymethyl ester (10 μM). During the Mn^{2+} -tracing of 600 seconds, Mn^{2+} (0.2 mM) was added to cells at 50 seconds. Agouti (100 nM) was added at 100 seconds. To study the effect of agouti on KCl-induced Ca^{2+} -influx, after the addition of agouti, the tracing was continued until 400 seconds when different concentrations of KCl were added. At the end of each experiment, digitonin (50 μM) was added at 500 seconds to determine the basal fluorescence level. The difference between this and the initial value was taken as 100 arbitrary fluorescence units (8,21). Mn^{2+} quench rate was estimated by linear regression of data collected after addition of each agonist or vehicle control (33). Insulin release experiment was conducted as described in the figure legend of figure 8. Data are the Mean $\pm$ SEM for 7 experiments in A, 3-4 experiments in B and 5 experiments in C. * $p < 0.05$ vs. Control.



0.001). This indicates Ca^{2+} -influx via Mn^{2+} -permeable Ca^{2+} -channels into HIT-T15 cells in response to 100 nM agouti.

To further investigate the role of agouti in stimulating insulin release in pancreatic β -cells, HIT-T15 cell were pretreated with 100 nM agouti and then challenged with different concentrations of KCl. As shown in Figure 9B, HIT-T15 cells responded to KCl, which causes membrane depolarization and subsequent activation of voltage-gated Ca^{2+} -channel, with a predictable dose-responsive increase in Mn^{2+} -influx. Moreover, the presence of agouti sensitized these cells to stimulation with KCl, resulting in a significantly more profound increase in Mn^{2+} -quench rate starting from 15 mM to 50 mM KCl compared to non-agouti-treated cells ($p < 0.05$ vs. control; Figure 9B).

The consequence of agouti potentiation of KCl's effect on Ca^{2+} -influx, as indicated by Mn^{2+} -quench, was studied by measuring insulin release from HIT cells under same conditions. As shown in Figure 9C, 10 mM KCl alone elicited no increase in insulin release from HIT cells compared to 5 mM KCl. However, the addition of 100 nM agouti into either 5 or 10 mM KCl resulted in 43 % and 51 % increase in insulin release, respectively ($p < 0.05$). KCl stimulated insulin release dose-dependently from 15 to 50 mM. Agouti potentiated this KCl effect on insulin release in these cells, with more apparent effects at higher KCl concentrations (25 and 50 mM, Figure 9C).

To evaluate the relevance of these data to humans, we determined agouti's effect on both Ca^{2+} -influx and insulin release in primary cultured human pancreatic islets. Ca^{2+} -influx into pancreatic islets was measured using the Mn^{2+} -quench method. 100 nM agouti induced a marked Mn^{2+} -influx in primary cultured human pancreatic islets (Figure 10A), indicating Ca^{2+} -influx into cells through Mn^{2+} -permeable Ca^{2+} -channels, similar to that

found in HIT-T15 cells. Agouti treatment of human pancreatic islets resulted in a 5-fold increase in Mn^{2+} -quench rate compared to vehicle control (Figure 10B, $p < 0.001$). In addition, agouti treatment also potentiated the effect of KCl in Ca^{2+} -influx in human pancreatic islets, as 25 mM KCl stimulated a more profound Mn^{2+} -influx into agouti-treated islets than non-treated islets (Figure 10A). This resulted in an increase in $[Ca^{2+}]_i$ (58 ± 11 nM over baseline) which was blocked by nitrendipine. The effect of agouti in stimulating insulin release was also studied in human pancreatic islets. As shown in Figure 11A, agouti (100 nM) and KCl (10 mM) induced a moderate increase in insulin release in the absence of glucose ($p < 0.05$). Under these conditions, agouti slightly potentiated KCl-induced insulin release, similar to that found in HIT-T15 cells. In the presence of 10 mM glucose, agouti stimulated a similar, albeit smaller, increase in insulin release ($p < 0.05$). As expected, KCl-induced insulin release was greatly potentiated by glucose ($p < 0.05$ vs. control). This effect was further significantly potentiated by agouti ($p < 0.05$ vs. control and KCl). Figure 11B demonstrates that agouti increases insulin release from human pancreatic islets over a broad range of glucose concentration (0-30 nM). This effect was blocked by 30 μ M nitrendipine (Figure 11C).

Because agouti is a paracrine factor that does not enter general circulation, we next examined the possibility that human express *agouti* in pancreas. Using Reverse Transcriptase-PCR, we detected an expected *agouti* fragment in human pancreas corresponding to the open reading frame of the human *agouti* gene (15) (Figure 12A). This was confirmed by Southern blot (Figure 12B).

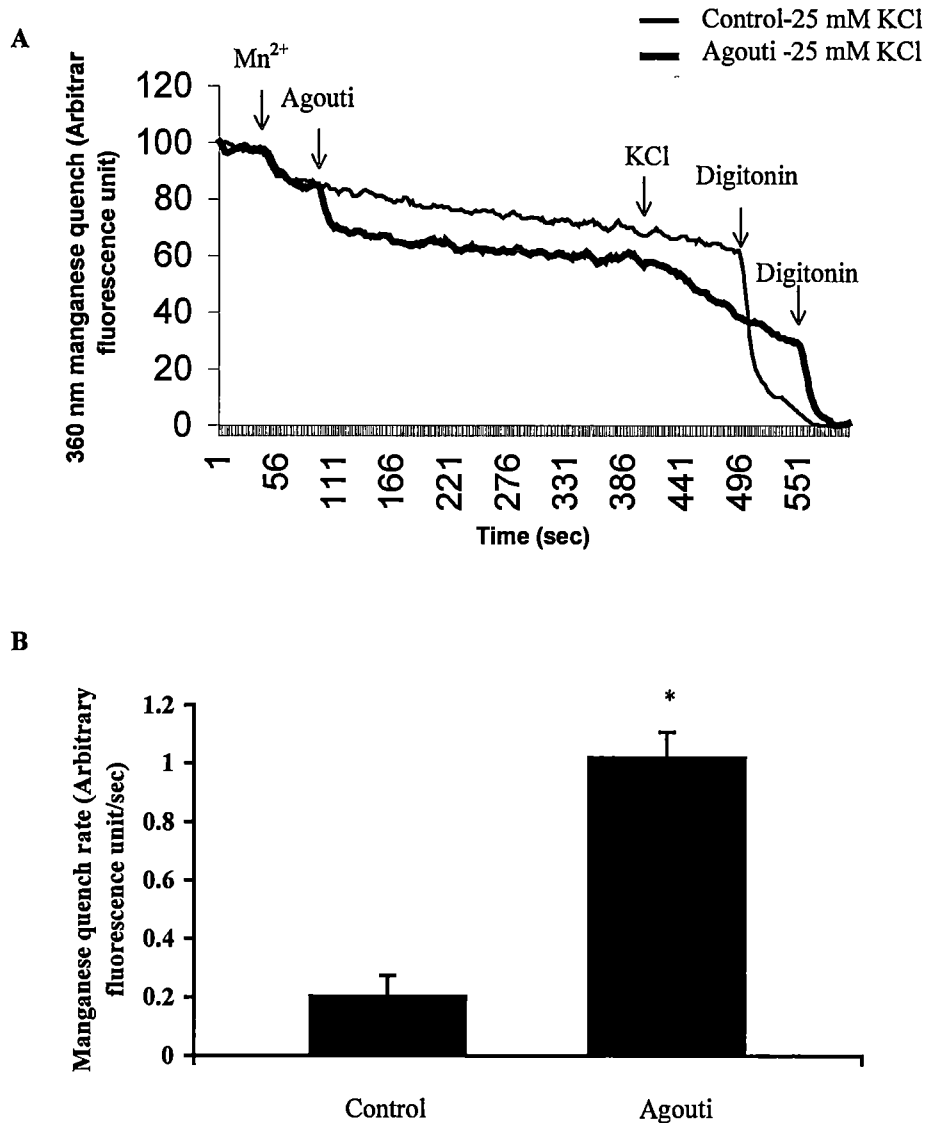
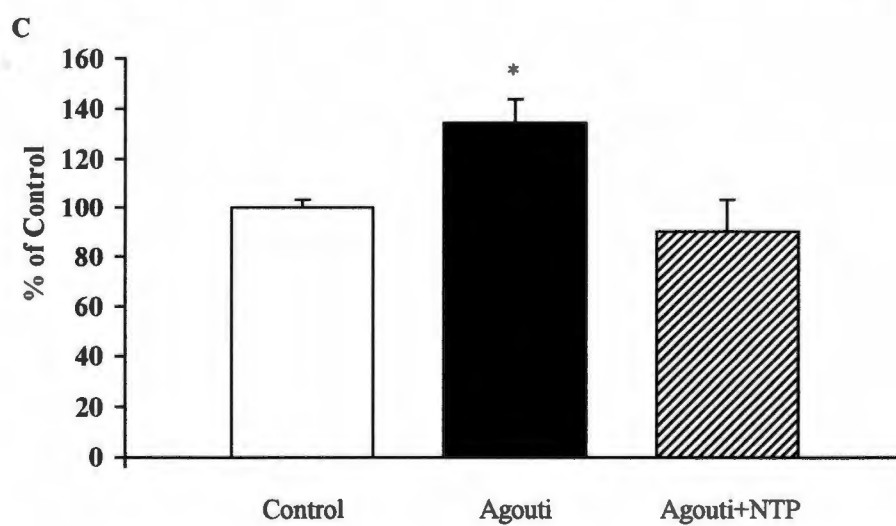
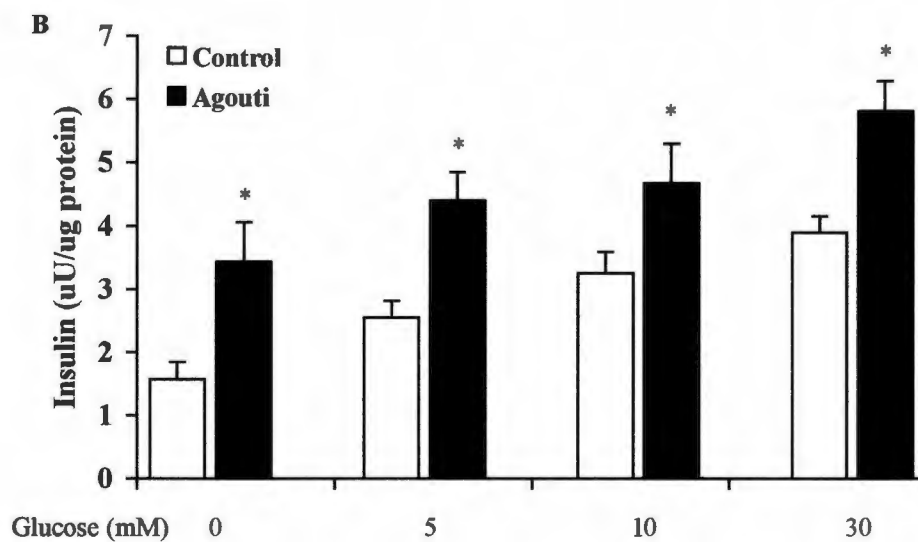
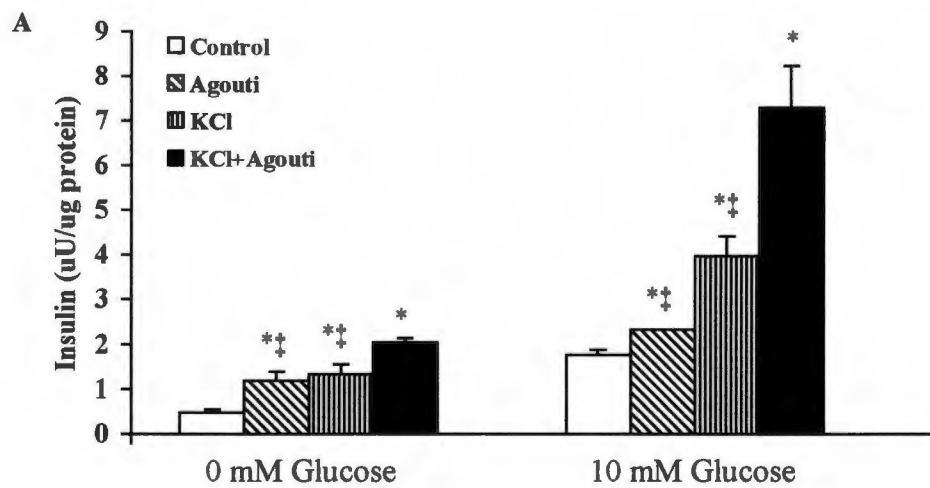


Figure 10. Effect of the agouti protein (100nM) on Ca²⁺-influx in primary cultured human pancreatic islets. A: Effects of agouti on Ca²⁺-influx in response to agouti and KCl in human pancreatic islets in the absence of glucose. MnCl₂, agouti, KCl and digitonin were added at times designated by arrows. Ca²⁺-influx was measured by the Mn²⁺-quench technique as described in the figure legend of Figure 9. B: Effects of agouti on Ca²⁺-influx in human pancreatic islets in the absence of glucose. Data are the Mean $\pm$ SEM for 4 experiments. * $p < 0.05$ vs. Control.

Figure 11. Effect of agouti protein on insulin release in human pancreatic islets. A: Effects of agouti protein (100 nM) and KCl (10 mM) on insulin release in human pancreatic islets both in the absence of glucose and in the presence of 10 mM glucose. Data are the Mean $\pm$ SEM for 3 experiments. * $p < 0.05$ vs. Control. ‡ $p < 0.05$ vs. KCl + Agouti. B: Effects of agouti protein (100 nM) on insulin release in the presence of different concentrations of glucose in human pancreatic islets. Data are the Mean $\pm$ SEM for 5 experiments. * $p < 0.05$ vs. Control. C: Agouti-induced insulin release in human pancreatic islets is blocked by Ca^{2+} -channel antagonist, nitrendipine (30 μM). Data are the Mean $\pm$ SEM for 4 experiments. Insulin release experiment was conducted as described in figure legend of Figure 8. * $p < 0.05$ vs. Control.



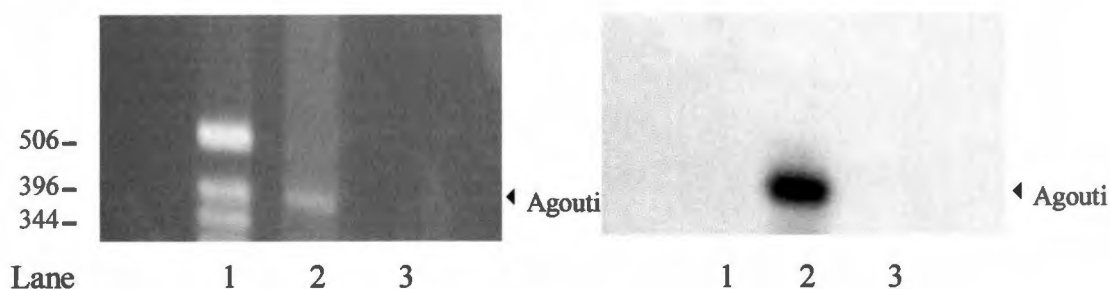


Figure 12. Detection of the expression of *agouti* gene in human pancreas using reverse transcriptase-PCR (RT-PCR). A: 1 % agarose gel electrophoresis of the RT-PCR products. 500 ng of human pancreas mRNA was reverse transcribed using random hexamer and then PCR-amplified using primers corresponding to the 5' and 3' ends of the putative open reading frame of *agouti* gene (27). The PCR conditions were: 1 cycle (94°C 5 minutes), 35 cycles (94°C 30 seconds, 65°C 1 minute and 72°C 1 minute), and 1 cycle (72°C 7 minutes). Lane 1, DNA molecular weight marker; lane 2, human pancreas with reverse transcriptase; lane 3, human pancreas without reverse transcriptase. B: Autoradiograph of the Southern blot. The gel shown in Figure 12A was transferred to nylon membrane and hybridized with specific human *agouti* cDNA probe at 68°C overnight, and was washed first with 2XSSC/0.1 % SDS at room temperature for 30 minutes followed by 0.1XSSC/0.1 % SDS at 65°C for 1 hour. The autoradiograph was exposed at -80°C for 30 minutes. Lane 1, 2 and 3 are the same as indicated in panel A.

V. DISCUSSION

The human homologue of the murine *agouti* gene, which causes an adult onset obesity/hyperinsulinemia syndrome in dominant mutants, is expressed in human adipocytes (15) and has been shown to stimulate lipogenesis in both human and murine adipocytes via a Ca^{2+} -dependent mechanism (12-14). However, transgenic mice which express *agouti* in adipose tissue under the control of the aP2 promoter do not become obese, unless hyperinsulinemia is concomitantly induced (18). Moreover, hyperplasia of pancreatic β cells and hyperinsulinemia precede the development of obesity in *agouti* mutant mice (19), further suggesting a critical role for hyperinsulinemia in *agouti*-induced obesity.

In addition, insulin resistance is a marked feature in both type II diabetes and obesity, and is a characteristic of *agouti* mutant mice (1,2). Although this resistance may result from either insulin receptor or post receptor defects, we have not found recombinant *agouti* protein to exert any effect on insulin signaling in skeletal muscle cells (unpublished data). However, it is well documented that hyperinsulinemia may directly down-regulate the insulin receptor (36), thereby leading to insulin resistance. This suggests that *agouti*-mediated hyperinsulinemia may also contribute to the insulin resistance in this animal model.

Insulin release can be stimulated by both nutrient and non-nutrient secretagogues, and $[\text{Ca}^{2+}]_i$ plays an important role in the signaling pathway for insulin release (21,37). The primary physiological insulin secretagogue is glucose. Normally, glucose oxidation increases the ATP/ADP ratio and inhibits ATP-sensitive K^+ -channels in β cells. Closing of K^+ -channels leads to depolarization of β cells and secondary activation of voltage-

dependent Ca^{2+} -channels and subsequent Ca^{2+} -influx into pancreatic β -cells, triggering insulin release (21). In addition, growing evidence indicates a K^{+} -ATP channel independent mechanism in glucose-stimulated insulin release (38,39). Gembal et al reported that opening K^{+} -ATP channel by diazoxide blocked the effect of glucose on mouse pancreatic β -cell insulin release, but did not prevent glucose potentiation of 30mM KCl-induced insulin release (38,39). This effect was not accompanied by changes in β -cell membrane potential, ^{45}Ca flux or $[\text{Ca}^{2+}]_i$ (38,39). However, it did require an initial elevation of $[\text{Ca}^{2+}]_i$, although further changes in $[\text{Ca}^{2+}]_i$ was not needed. This effect was not ascribed to phosphoinositide metabolism or activation of protein kinase A or C. Instead, it required glucose metabolism and was correlated with β -cell ATP/ADP ratio (39). This suggests that in addition to its primary signal in stimulating insulin release (i.e. closure of K^{+} -ATP channels-depolarization- Ca^{2+} influx), glucose can also enhance insulin release by modulating and amplifying the secretory response to increased $[\text{Ca}^{2+}]_i$ signal while it did not further affect changes in membrane potential and $[\text{Ca}^{2+}]_i$. This mechanism appears to involve changes in β cell energy state (39).

Our data demonstrate that 100 nM agouti induced a gradual and sustained increase of $[\text{Ca}^{2+}]_i$ in pancreatic β cells in the absence of glucose. Agouti also stimulated significant insulin release in these cells in the absence of glucose. This suggests that agouti stimulation of insulin release is not dependent on glucose. We also studied the effect of agouti on glucose-induced insulin release in HIT-T15 cells. Our data showed that the half-maximal effect of glucose in insulin secretion in HIT cells is approximately 2.5 mM and reaches plateau at 5 mM, similar to previous report (40). The half-maximal effect of glucose in insulin release in human pancreatic islets is approximately 7 mM. As

the half-maximal dose of glucose is 8 mM for isolated pancreatic islets (41), the cell response to glucose is exaggerated in HIT-T15 cells (40). We showed that agouti stimulated a marked insulin release both in the absence and presence of different concentrations of glucose, suggesting that agouti is a potent insulin secretagogue.

However, we found that agouti induced a smaller increase in $[Ca^{2+}]_i$ in the presence than in the absence of glucose. Several reports revealed that glucose has both a stimulatory and an inhibitory effect on both ^{45}Ca efflux and insulin release (reviewed in 42). This inhibitory effect, however, is more obvious when Ca^{2+} entry into β cells is prevented by using diazoxide (43) or removal of extracellular Ca^{2+} (44). This has been attributed to the ability of glucose to promote intracellular Ca^{2+} sequestration. However, whether our observation also results from this effect of glucose is unclear, as the static incubation method used in our study made it impossible to further study this phenomenon.

The observed increase in $[Ca^{2+}]_i$ may result either from the release of Ca^{2+} from intracellular stores or from Ca^{2+} -influx. Previous data from this laboratory showed that agouti stimulation of lipogenesis was blocked by nitrendipine, suggesting a Ca^{2+} -influx-mediated effect (12). Moreover, we have shown that agouti-induced increase in $[Ca^{2+}]_i$ is inhibited by Ca^{2+} -channel antagonism in several cell types (6,11). In addition, the structure of the C-terminal region of agouti protein exhibits structural homology to the ω -conotoxins and the plectoxins, two toxins produced by cone snail *Conus geographus* and hunting spider *Plecteurys tristis*, respectively, which primarily affect Ca^{2+} -channel function (45). Further, our data using the Mn^{2+} -quench technique clearly demonstrated

that agouti-stimulated Mn^{2+} -influx into both HIT-T15 pancreatic β -cells and human pancreatic islets, indicating Ca^{2+} -influx into these cells in response to agouti stimulation.

This Ca^{2+} -influx resulted in increases in both basal and glucose-stimulated insulin release in these cells, demonstrating that agouti is an insulin-secretagogue in both pancreatic β -cell lines and human pancreatic islets. Moreover, we have shown here that agouti is expressed in human pancreas. As agouti is a paracrine factor which does not enter general circulation (46), these data suggest that agouti may contribute to the development of hyperinsulinemia in humans in a paracrine fashion, similar to its paracrine actions in adipocytes (12) and hair follicles (3).

In addition to stimulation of basal insulin release, agouti also potentiated the effect of KCl on insulin release in both HIT-T15 cells and human pancreatic islets. Under basal (glucose-free) conditions, agouti potentiation of KCl-induced insulin release is only apparent at high KCl concentrations. In the presence of 10 mM glucose, however, the effect of a low concentration of KCl, 10 mM, on insulin release was significantly potentiated by agouti. This suggests that agouti potentiation of KCl-induced insulin release is dependent on glucose. As glucose has been shown to modulate β cell secretory response to increased $[Ca^{2+}]_i$ stimulation (38,39), the glucose dependence of agouti potentiation on KCl-induced insulin release may also involve mechanisms that require glucose to sensitize β cell response to the primary stimulant. However, we also found that agouti potentiated KCl-induced Ca^{2+} -influx into pancreatic β -cells, suggesting that agouti may modulate Ca^{2+} -channel activity, thereby sensitizing Ca^{2+} -channel response to other stimuli. This mechanism, combined with glucose-dependent amplification of β cell

secretory machinery to the primary $[Ca^{2+}]_i$ signal, may contribute to agouti potentiation of KCl-induced insulin release in the presence of glucose.

Non-nutrient insulin secretagogues may modulate β cell ion-channels (K^+ -channels or Ca^{2+} -channels) by acting through cAMP or phospholipase (PL) pathways, activating protein kinase A (PKA) or protein kinase C (PKC), or increase the release of arachidonic acid (AA), thereby sensitizing these channels to primary stimuli. Alternatively, they may phosphorylate target proteins involved in secretory machinery, thereby amplifying cell response to increased $[Ca^{2+}]_i$ signal (reviewed in 37).

It is not clear whether the effect of agouti is similarly mediated by such mechanisms or is a direct effect on Ca^{2+} -channels. However, the homology between the C-terminal region of agouti and ω -conotoxins and plectoxins suggests that agouti may act directly on Ca^{2+} -channels and thereby modify channel activity (45).

It is well documented that agouti antagonizes melanocortin receptors and inhibits the generation of cAMP during its regulation of pigment production (3,4). Whether agouti also inhibits cAMP production in pancreatic β cells is not clear, as neither cAMP nor other second messengers have been determined in our study. Consequently, the mechanisms involved in agouti potentiation of KCl-induced insulin release remains to be further explored.

Both central and peripheral effects of agouti in agouti-induced obesity have been extensively explored. It was recently reported that MC4-R knockout mice developed an obesity/hyperinsulinemia syndrome similar to that found in agouti mutants (5). In addition, intracerebroventricular administration of MC4-R agonist, MTII, inhibited feeding, while this effect was prevented by co-administration of SHU9119, a potent

MC4-R antagonist (47). These data indicate an important role for central MCR in regulating feeding behavior, suggesting that central MC4-R antagonism may be one of the mechanisms involved in agouti-induced obesity.

Recent data showed that central administration of SHU9119 prevented the anorexic effect of leptin, suggesting that MC4-R may be involved in leptin signaling (48).

However, Boston et al studied the effect of agouti and leptin deficiency on weight gain in agouti mutant (lethal yellow, A^y/a), leptin knockout (lep^{ob}/lep^{ob}) mice and double mutant ($A^y/a lep^{ob}/lep^{ob}$) mice. Agouti produced similar weight gain in both the wild type and lep^{ob}/lep^{ob} mice, demonstrating that the effect of agouti and leptin deficiency in inducing obesity are independent (49). Further, The leptin resistance seen in obese yellow mice is due to desensitization of the leptin receptor, not due to a defect in leptin signaling, as removal of leptin from these mice restored leptin sensitivity (49). These data suggest that agouti-induced obesity is independent of leptin action and that the increased level of leptin in agouti mutant may serve to attenuate agouti-induced obesity.

Alternatively, data in this report and in our previous reports suggest an important role for peripheral effect of agouti in agouti-induced obesity. Insulin is well known to stimulate lipogenesis and inhibit hormone-induced lipolysis. Similarly, we have demonstrated that agouti not only promotes adipocyte lipogenesis (12,13), but also inhibits lipolysis (50). Both murine and human adipocytes express high levels of MC2-R (16,51), whose natural ligand is the potent lipolytic agonist ACTH. Recent data showed that increasing $[Ca^{2+}]_i$ in rat adipocytes dose-dependently inhibited hormone-stimulated lipolysis (52). In addition, we have recently demonstrated that recombinant agouti protein inhibited lipolysis in human adipocytes via a Ca^{2+} -dependent mechanism (50). Thus,

agouti appears to exert insulin-like effects on adipocytes. These effects, combined with agouti-induced insulin release, may then act synergistically on adipocytes and thereby contribute to the agouti induced obesity syndrome. In addition, hyperinsulinemia may contribute to insulin resistance by direct down-regulation of insulin receptor (36). This indicates that agouti-stimulation of insulin release may also play an important role in the development of insulin resistance in agouti mutant mice.

In summary, our data indicate that agouti acts directly in pancreatic β cells and may thereby play an important role in the hyperinsulinemia of dominant *agouti* mutations. This hyperinsulinemia may act in an additive or synergistic manner with adipocyte *agouti* expression to increase adipocyte triglyceride accumulation; it may also contribute to the insulin resistance seen in agouti-induced obesity syndrome.

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PART 4

THE AGOUTI GENE PRODUCT STIMULATES ISLET AMYLOID POLYPEPTIDE (AMYLIN) SECRETION IN PANCREATIC β -CELLS¹

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I. ABSTRACT

Overexpression of the murine *agouti* gene results in obesity, hyperinsulinemia and type II diabetes. We have shown the human homologue of *agouti* to regulate human adipocyte metabolism and lipid storage via a Ca^{2+} -dependent mechanism. We have also demonstrated *agouti* expression in human pancreas, and that agouti stimulates insulin release via a similar Ca^{2+} -dependent mechanism. Plasma amylin is also elevated in agouti mutant mice. Amylin is co-secreted with insulin from β -cells, and overexpression of human amylin in β -cells in agouti mutant mice resulted in accelerated pancreatic amyloid deposition, severely impaired β -cell function and a diabetic phenotype. We report here that agouti protein stimulates amylin release in both the HIT-T15 β -cell line and human pancreatic islets in the presence of a wide range of glucose concentrations (0-16.7 mmol/L), similar to its effect on insulin release; this effect was blocked by 30 $\mu\text{mol/L}$ nitrendipine, confirming a Ca^{2+} -dependent mechanism. Accordingly, agouti stimulation of amylin release may serve as a compensatory system to regulate blood glucose in agouti mutants.

II. INTRODUCTION

Ubiquitous expression of the mouse *agouti* gene results in a yellow coat color, as well as obesity, hyperinsulinemia, insulin resistance and type II diabetes (1).

The human homologue of *agouti* is expressed in adipose tissue (2). We have previously shown that recombinant agouti protein coordinately regulates adipocyte lipogenesis and lipolysis, thereby promoting lipid storage in adipose tissue (3,4). We have also demonstrated that agouti is expressed in human pancreas, and stimulates both Ca^{2+} -signaling and insulin release in human pancreatic islets (5), thereby contributing to hyperinsulinemia.

Islet amyloid polypeptide, also called amylin, is co-localized with insulin in β -cell secretory granules (6) and co-secreted with insulin in response to glucose and non-glucose secretagogues (7). Recent data showed that amylin delayed the postprandial rise in plasma glucose levels by slowing the rate of gastric emptying (8), suggesting that amylin, as another β -cell hormone, may coordinate with insulin in regulating blood glucose homeostasis under physiological conditions.

However, amylin is the major component of islet amyloid deposits, which usually accumulate extracellularly between β -cells and capillaries in the islets of most of the type II diabetic patients (9), and lead to diminished β -cell mass, impaired β -cell function and hyperglycemia in the later stage of type II diabetes. Plasma amylin levels are elevated in *agouti* mutants compared to wild type mice (10). Further, overexpression of human amylin in the pancreas of *agouti* mutants results in accelerated islet amyloid deposition, severely impaired β -cell function and diabetic phenotype (11). Since amylin release is

regulated similarly to that of insulin, it is possible that agouti may directly stimulate amylin release, similar to its effect on insulin release.

Consequently, the present study was designed to investigate the effect of recombinant agouti protein on amylin release in pancreatic β -cells. We report here that recombinant agouti protein stimulated amylin release in both HIT-T15 pancreatic β -cell line and human pancreatic islets via a Ca^{2+} -dependent mechanism, similar to its effect on insulin release and adipocyte metabolism (3-5).

III. MATERIALS AND METHODS

A. Isolation and purification of human pancreatic islets

Human pancreas was obtained from multiorgan donors and transported in cold University of Wisconsin solution (5). Islets were isolated using collagenase digestion and Ficoll gradient centrifugation as previously described (5). Briefly, the pancreas was distended with collagenase type V (0.5 mg/ml) –type XI (2 mg/ml)(Sigma, St. Louis, MO) in Hank's balanced salt solution and digested in the same solution at 37°C with shaking for 30 min. The tissue digest was then washed and sifted through a 500 µm nylon filter and purified by Ficoll gradient centrifugation (Fluka, Milwaukee, WI). Purified islets were maintained in RPMI-1640 medium supplied with 10 % fetal bovine serum (FBS, Hyclone, Logan, UT) and antibiotics (50 U/ml of penicillin and 5 µg/ml of streptomycin) for 48 hrs. The use of human pancreas was approved by the University of Tennessee Institutional Review Board and by Tennessee Donor Services.

B. Cell Culture

HIT-T15 pancreatic β -cell (American Type Culture Collection, Rockville, MD) was cultured in F-12K nutrient mixture supplemented with 10 % horse serum, 2.5 % FBS and antibiotics. Cell culture reagents were obtained from Life Technologies (Grand Island, NY) except for those mentioned above.

C. Recombinant agouti production

Recombinant agouti polypeptide was produced by subcloning agouti cDNA into a baculoviral expression vector, which was then used to infect *Trichipphisia ni* (*T. ni*) cells (4,5). Medium containing the secreted agouti was collected 48 hrs post-infection and purified as previously described (4,5).

D. Amylin release experiment

Amylin release experiments were conducted using a similar procedure to that previously described for insulin release (5). Briefly, HIT-T15 pancreatic β -cells and human pancreatic islets were incubated in glucose-free, serum-free medium for 2 hrs on the day of experiment and then incubated with test agents indicated under each figure for 2 hrs. Medium was then removed and stored at -80°C for amylin measurement. Cells were sonicated in sucrose buffer (250 mmol/L sucrose, 1 mmol/L dithiothreitol (DTT), 0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF) and 1 mmol/L ethylenediaminetetraacetic acid (EDTA), Sigma, St. Louis, MO) and stored at -80°C for protein measurement.

Amylin level in the medium was measured by a human RIA kit (Peninsular, Belmont, CA). The antibody in the kit has been shown to cross-react with both human and hamster amylin (7). The sensitivity of the assay is 2 pg/tube and the coefficient of variation is less than 10 %. The total amount of protein in the cells/islets was determined by a modified Bradford method using Coomassie blue dye (Pierce, Rockford, IL).

E. Statistics

All data are expressed as mean $\pm$ SEM. Data were analyzed by One-Way Analysis of Variance (ANOVA) using the procedures of the SPSS Inc. (Chicago, IL). A p value < 0.05 is considered significant.

IV. RESULTS

As shown in Figure 13A, glucose dose-dependently simulated amylin release in HIT-T15 pancreatic β -cells (39 % and 98 % increase at 2.5 and 10 mmol/L glucose, respectively, $p < 0.05$), with a stimulatory concentration as low as 2.5 mmol/L. The addition of 100 nmol/L agouti further enhanced amylin release in these cells, with 34 %, 79 % and 34 % further increase at 0, 2.5 and 10 mmol/L glucose concentrations, respectively ($p < 0.05$).

Figure 13B shows that agouti-stimulated amylin release in HIT-T15 cells was blocked by 30 μ mol/L nitrendipine, suggesting that this effect of agouti is mediated via a Ca^{2+} -dependent mechanism, similar to our previous reports of agouti regulation of adipocyte metabolism and β -cell insulin release (3-5).

Similar results were observed in primary cultured human pancreatic islets (Figure 14). However, in contrast to HIT-T15 cells, glucose exerted its stimulatory effect on amylin release at a higher concentration, 16.7 mmol/L, and 100 nmol/L agouti further enhanced amylin release under these glucose concentrations ($p < 0.05$, Figure 14A). Such differences between pancreatic β -cell line and primary cultured pancreatic islet responses to glucose have been previously documented (12). Agouti-stimulated amylin release in human pancreatic islets was blocked by nitrendipine (Figure 14B), similar to the observed effects in HIT-T15 cells noted above.

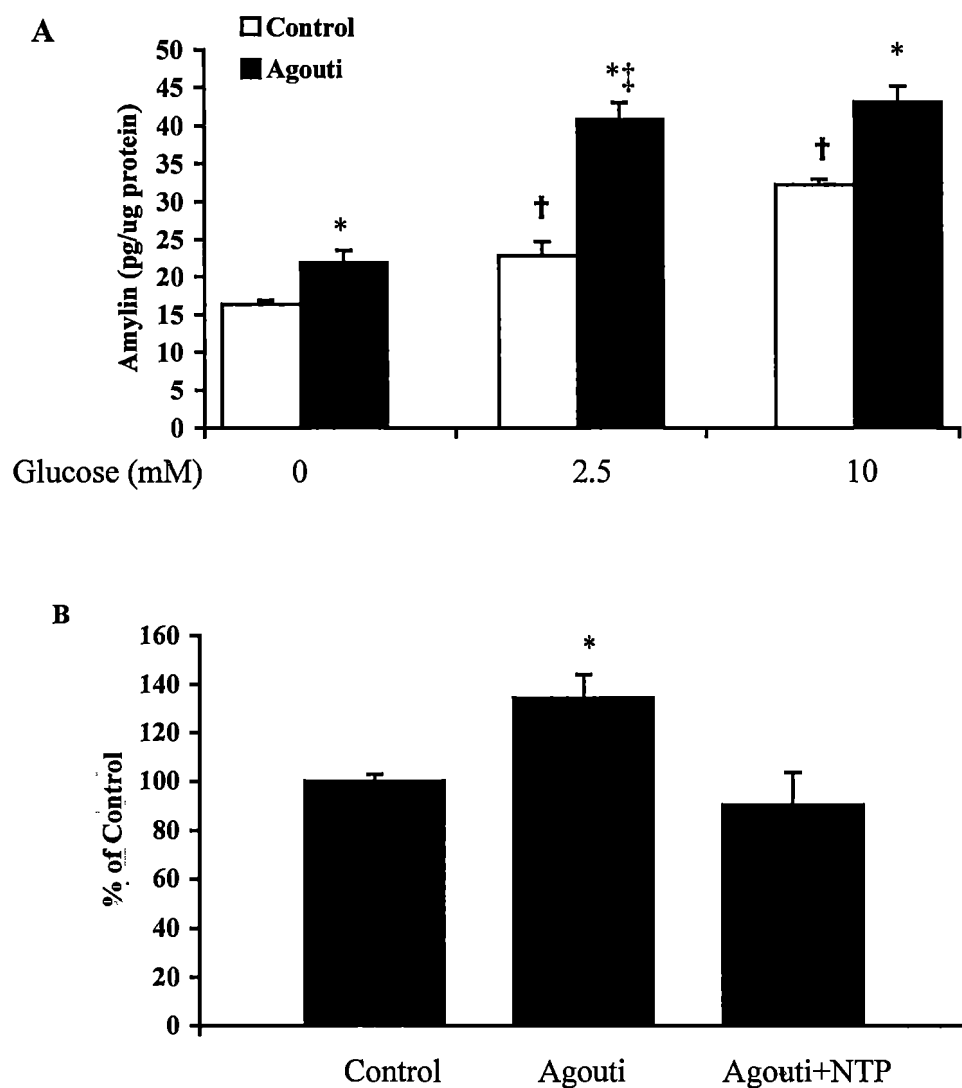


Figure 13. Effects of recombinant agouti protein on amylin release in HIT-T15 pancreatic β -cell line. HIT-T15 cells were pretreated with glucose-free, serum-free medium for 2 hrs. Cells were then incubated in the same medium with agouti (100 nmol/L) in the presence of different concentrations of glucose (0-10 mmol/L) (A) or with agouti (100 nmol/L) and nitrendipine (30 μ mol/L) in the absence of glucose (B) for 2 hrs. At the end of incubation, medium was removed and stored at -80°C before amylin measurement. Cells were sonicated and stored at -80°C for protein correction. Data are means $\pm$ SEM for 3-4 experiments. A: * $p < 0.05$ vs. control, † $p < 0.05$ vs. previous glucose treatment. ‡ $p < 0.05$ vs. previous agouti treatment. B: * $p < 0.05$ vs. control and Agouti + NTP.

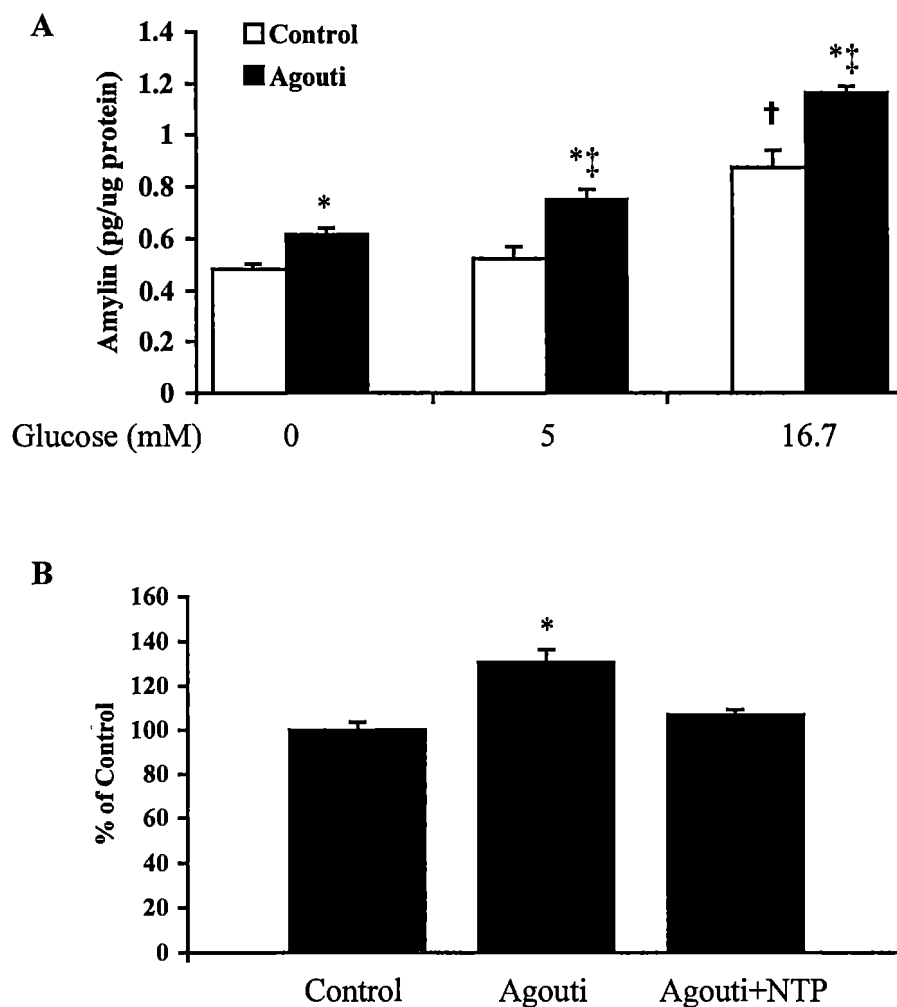


Figure 14. Effect of recombinant agouti protein on amylin release in human pancreatic islets. Human pancreatic islets were isolated via a collagenase digestion and Ficoll gradient centrifugation method. Islets were pretreated with glucose-free, serum-free medium for 2 hrs. Islets were then incubated in the same medium with agouti (100 nmol/L) in the presence of different concentrations of glucose (0-16.7 mmol/L) (A) or with agouti (100 nmol/L) and nitrendipine (30 μ mol/L) in the absence of glucose (B) for 2 hrs. At the end of incubation, medium was removed and stored at -80°C before amylin measurement. Islets were sonicated and stored at -80°C for protein correction. Data are means $\pm$ SEM for 3-4 experiments. A: * $p < 0.05$ vs. control, † $p < 0.05$ vs. previous glucose treatment. ‡ $p < 0.05$ vs. previous agouti treatment. B: * $p < 0.05$ vs. control and Agouti + NTP.

V. Discussion

Amylin is a normal β -cell hormone whose basal plasma level is about 1-10 pmol/L, and reaches peak value of 8-20 pmol/L after meals (reviewed in 13,14). Previous data have shown that the plasma amylin level is elevated in A^{vy} mice (10). In addition, amylin level is also elevated in patients with early, diet-controlled Type II diabetes and in obese individuals while being decreased in patients in the later stages of type II diabetes (reviewed in 13,14). The concurrence of hyperamylinemia and hyperinsulinemia in these conditions may be explained by the similar nutritional and hormonal regulation in both insulin and amylin secretion (7).

In the present study, we have demonstrated that agouti, the product of a murine obesity gene, directly stimulated amylin release in both pancreatic β -cell line and human pancreatic islets in the presence of a wide range of glucose concentrations. Therefore, agouti stimulation of amylin release may contribute to the elevated amylin level present in agouti mutant mice (10). In addition, we have recently shown that agouti is expressed in human pancreas (5). As agouti is a paracrine factor that does not enter the general circulation (15), these data suggest that agouti may stimulate amylin release in humans in a paracrine/autocrine fashion, similar to its paracrine/autocrine actions on β -cell insulin release (5) and adipocyte lipid metabolism (3,4).

Recently, several studies have demonstrated that administration of amylin dose-dependently delayed the rise in plasma glucose levels after oral glucose load by slowing the rate of gastric emptying, with an ED_{50} of 20 pmol/L (8). Amylin also dose-dependently suppressed arginine-induced glucagon secretion in rats, with an EC_{50} of 18 pmol/L (16). As these effects occur at close to physiological concentrations of amylin, it

is hypothesized that amylin may serve to delay glucose input into blood and inhibit hepatic glucose output after meal, thereby coordinating with insulin to modulate blood glucose level under physiological conditions. Consistent with this, the human amylin analogue pramlintide has been shown to improve postprandial glycaemic control in both type I and II diabetic patients (17). In addition, amylin has been shown to suppress food intake in rats and mice after either peripheral or central administration (18,19). Thus, amylin may also act as a peripheral satiety factor to modulate food intake, as do insulin, cholecystokinin and glucagon-like peptide-1.

Consequently, agouti stimulation of amylin release may serve as a compensatory mechanism along with insulin in regulating blood glucose homeostasis. Moreover, agouti stimulation of amylin release may also act as a peripheral satiety factor to modulate food intake, thereby representing a feedback mechanism in body weight regulation.

However, the association of islet amyloid deposition with β -cell failure indicates that amylin may have an important role in the pathogenesis of type II diabetes. Because of the difference in the composition at amino acids 20 and 29, amylin from rodents is not amyloidogenic, whereas islet amyloid formation does occur in humans, cats and racoons (20).

Although humans express an amyloidogenic species of amylin, amyloid deposition rarely occurs in non-diabetic individual (9). In addition, most lines of transgenic mice overexpressing human amylin in β -cells do not spontaneously develop islet amyloid deposits and hyperglycemia (21-23). One strain of homozygotic transgenic mice overexpressing human amylin did develop hyperglycaemia (24). However, this is associated with small intra- and extracellular amorphous IAPP aggregates resulting in

cytotoxicity and β -cell death, instead of large extracellular amyloid deposits that are commonly seen in most of the transgenic animals and type II diabetes patients. In addition, two recently developed immortal mouse pancreatic β -cell lines overexpressing human amylin also do not spontaneously develop islet amyloid (25). On the other hand, introducing the *A^y* trait, which is characterized by obesity and insulin resistance, into these transgenic animals did result in extensive islet amyloid formation, leading to reduced β -cell mass and type II diabetes (11). Similarly, feeding these transgenic mice a high-fat diet, or introducing the *ob* trait into these animals, both of which induce insulin resistance, similarly resulted in accelerated amyloidogenesis and profound hyperglycemia (26,27).

These data suggest that production of an amyloidogenic amylin by itself is necessary but not sufficient to induce amyloid deposit formation, whereas the interaction between obesity/insulin resistance status and amylin expression appears to be important in islet amyloidogenesis. Islet amyloid formation may be both a consequence of obesity/insulin resistance and a cause of β -cell failure and subsequent hyperglycemia in the later stage of type II diabetes.

Our data demonstrated that agouti stimulates amylin release from human pancreatic islets. This agouti-induced hyperamylinemia may predispose individuals to the development of islet amyloid deposit, which, under conditions that induce obesity/insulin resistance status, may accelerate the process of amyloidogenesis, and ultimately result in hyperglycemia and type II diabetes. In addition, we have shown agouti to stimulate insulin release, thereby contributing to hyperinsulinemia (5). Further, we have shown that adipocyte agouti protein and mRNA levels are positively correlated to fatty acid synthase

(FAS, a key enzyme in *de novo* lipogenesis) activity and mRNA level in human adipose tissue (28), indicating a potential role for agouti in inducing human obesity *in vivo*. Thus, agouti-induced hyperamylinemia, combined with its effects in promoting obesity and hyperinsulinemia, may contribute to the risk factors that may lead to increased amyloid deposition and type II diabetes.

In conclusion, our data showed that recombinant agouti protein is a potent secretagogue for amylin release in pancreatic β -cells. This may serve as a compensatory regulation of blood glucose homeostasis. However, agouti stimulation of amylin release may also result in increased islet amyloid deposition, leading to impaired β -cell function, reduced β -cell mass and diabetic phenotype.

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PART 5

**THE AGOUTI GENE PRODUCT INHIBITS LIPOLYSIS IN HUMAN
ADIPOCYTES VIA A CA^{2+} -DEPENDENT MECHANISM¹**

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I. ABSTRACT

Overexpression of the murine *agouti* gene results in obesity. The human homologue of *agouti* is expressed primarily in human adipocytes, and we have shown recombinant *agouti* protein to increase adipocyte intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) and thereby stimulate lipogenesis. However, since recent data demonstrate that increasing adipocyte $[\text{Ca}^{2+}]_i$ may also inhibit lipolysis, we have now investigated the role of *agouti*-induced $[\text{Ca}^{2+}]_i$ increases in regulating lipolysis in human adipocytes. Short-term (1 hr) exposure to recombinant *agouti* (100nM) protein had no effect on basal lipolysis, although longer-term treatment (24 hr) caused a 60% decrease in basal lipolysis ($P < 0.0001$). Short-term *agouti* treatment totally inhibited ACTH-induced lipolysis ($P < 0.05$). Since melanocortin receptors (MCR) are involved in some actions of *agouti*, we next determined if *agouti*'s anti-lipolytic effect is exerted through competitive antagonism of the ACTH receptor (MCR-2). 1 μM forskolin, an adenylate cyclase activator, induced a 40% increase in lipolysis in human adipocytes ($P < 0.05$); this effect was reversed by 100nM *agouti* ($P < 0.005$), demonstrating that the anti-lipolytic effect of *agouti* is distal to the ACTH receptor. To determine the role of $[\text{Ca}^{2+}]_i$ in the anti-lipolytic effect of *agouti*, human adipocytes were treated with KCl or arginine vasopressin (AVP) to stimulate voltage and receptor-stimulated Ca^{2+} influx, respectively. Both agents caused inhibition of forskolin-induced lipolysis similarly ($P < 0.005$). Furthermore, *agouti*'s anti-lipolytic effect was also blocked by the Ca^{2+} channel blocker nitrendipine. These data demonstrate that *agouti* exerts a potent anti-lipolytic effect in human adipocytes via a Ca^{2+} -dependent mechanism. This effect, combined with *agouti*-induced lipogenesis,

represents a coordinate control of adipocyte lipid metabolism which may contribute to an agouti-induced obesity syndrome.

II. INTRODUCTION

The mouse *agouti* gene is normally involved in regulating coat color development (1). It is expressed only in the neonatal skin of wild-type mice during hair growth, such that the expression of *agouti* causes a transient switch from black (eumelanin) to yellow (phaeomelanin) pigment. This gives rise to black hairs with subapical yellow band (1,2). However, dominant mutations in the promoter region of this gene, such as viable yellow (A^y), leads to overexpression of the normal protein throughout life which results in not only an altered pattern of pigmentation, i.e. a yellow coat color, but also in a syndrome of adult-onset obesity and insulin resistance (3,4).

Agouti regulation of pigment development occurs via a paracrine action within the hair follicle (5,6), where agouti competitively antagonizes the binding of melanocyte stimulating hormone (α -MSH) to its receptor (melanocortin receptor-1, MCR-1). This results in an inhibition of cAMP production and a switch from eumelanin to phaeomelanin production (7). Agouti has been demonstrated to similarly antagonize other classes of MCRs (8). Consequently, this competitive antagonism of melanocortin binding has been considered by some investigators to serve as a paradigm for agouti's action in obesity.

However, the actual mechanism of the action of agouti in the development of obesity is not clear. Agouti protein was recently demonstrated to inhibit melanogenesis independent of any melanocortin ligand (9,10). Moreover, we have shown that agouti regulates several cellular functions, such as Ca^{2+} signaling, independent of melanocortin receptor antagonism (11). Furthermore, we have demonstrated that agouti-induced obesity is not antagonized by the melanocortin receptor agonist, NDP-MSH (12).

We have found that recombinant agouti protein increases intracellular calcium ($[Ca^{2+}]_i$) in several cell types(11), including both murine and human adipocytes. Agouti protein also stimulated lipogenesis in both murine and human adipocytes via a Ca^{2+} -dependent mechanism (13-15). Since the human homologue of *agouti* is expressed primarily in adipose tissue (16), and since $[Ca^{2+}]_i$ plays an important role in the metabolic disorders of obesity and insulin resistance (17-19), agouti may similarly act via a paracrine mechanism on adipocytes Ca^{2+} signaling and thereby stimulate lipogenesis.

Both basal and agonist-stimulated adipocyte lipolysis is impaired in agouti mutant mice compared to their wild-type littermates (20-22). This impairment may either be a consequence of the obesity syndrome or it may result from a direct inhibitory effect of agouti on adipocyte lipolysis. Recent data have demonstrated that increasing $[Ca^{2+}]_i$ inhibits lipolysis in a dose-dependent manner (23). Accordingly, it is possible that agouti may coordinately regulate both lipogenesis and lipolysis in adipocytes via Ca^{2+} -mediated mechanisms. Alternatively, two melanocortin receptors, MCR-2 and MCR-5, are expressed in adipocytes (24). MCR-5 is a ubiquitously expressed receptor, while MCR-2 is the adrenocorticotrophic hormone (ACTH) receptor, whose natural ligand (ACTH) is a potent lipolytic hormone (25). Thus, agouti may also exert an anti-lipolytic effect via antagonism of this melanocortin receptor.

Accordingly, the present study was conducted to determine the effect of recombinant agouti protein on human adipocyte lipolysis and address the role of both $[Ca^{2+}]_i$ and the ACTH receptor in this process. We report here that agouti inhibits basal and agonist-stimulated lipolysis in primary cultured human adipocytes. This action is

Ca²⁺-dependent, as it can be mimicked by KCl and blocked by the Ca²⁺-channel blockade nitrendipine, and is not dependent upon ACTH receptor antagonism.

III. Materials and Methods

A. Isolation and culture of human adipocytes

Human subcutaneous abdominal adipose tissue was obtained from patients undergoing elective abdominal cosmetic surgery. These patients were normal, with no known history of metabolic disorders. This protocol was approved by the Institutional Review Board for Human Subjects and the Committee for Research Participations of the University of Tennessee. Adipocytes were isolated as we have previously described (26). Briefly, the tissue was washed several times with Hank's Balanced Salt Solution, minced into small fragments and digested with type I collagenase (1mg/ml) in a shaking water bath at 37°C for 30-40 min. Cells were then filtered through a sterile nylon filter (500µm mesh), and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1% fetal bovine serum (FBS), 1% bovine serum albumin (BSA), 100 U/ml penicillin, 100µg/ml streptomycin and 50µg/ml gentamicin.

B. Production and purification of recombinant agouti polypeptide

A 614 bp XbaI/PstI fragment of the full-length mouse *agouti* cDNA (27) or human *agouti* cDNA (16) was subcloned into a baculovirus expression vector and expressed in *Trichiphsia ni* (*T.ni*) cells. Medium was collected 48 hours after infection, filtered through a Whatman 3 filter, and purified as previously described (28).

C. Lipolysis experiments

Human adipocytes were incubated in 24-well plates with different treatment for either 1 hour or 24 hours as indicated, and glycerol release into the culture medium was determined as an indicator for lipolysis using an one-step enzymatic fluorometric method (29). The media collected from 1 hr treatments were used directly for glycerol

measurements. For 24 hr treatments, media were removed, and cells were washed with Hank's Balanced Salt Solution and incubated in fresh medium for a further 30 min. This media were then used for glycerol measurements for 24 hr treatments. After medium was collected, HClO_4 was added to give a final concentration of 3% for deproteinization. The sample was centrifuged at $9000 \times g$ for 20 min. The supernatant was then collected and neutralized with 10N NaOH (23). Samples were stored at -80°C prior to glycerol assay.

D. Protein assay

Cells were collected, homogenized and stored at -80°C for subsequent total protein correction by a modified Bradford method using Coomassie Blue dye (Pierce, Rockford, IL).

E. Statistics

All data are expressed as mean $\pm$ SEM. Statistical analysis was performed by analysis of variance or Student t-test, as appropriate.

IV. RESULTS

To investigate the role of agouti on lipolysis, we first studied the effect of agouti on both basal and ACTH-induced lipolysis in human adipocytes. Short-term (1 hr) exposure of human adipocytes to recombinant agouti protein (100nM) had no effect on basal lipolysis. However, recombinant agouti protein completely blocked 50nM ACTH-induced lipolysis ($P < 0.05$, Figure 15). Similar results were obtained in 3T3-L1 murine adipocytes (data not shown).

Since agouti is a potent MCR antagonist (7), we next determined whether agouti's anti-lipolytic effect is exerted through competitive antagonism of the ACTH receptor (MCR-2). 1 μ M forskolin, an adenylate cyclase activator, induced a 40% increase in lipolysis in human adipocytes ($P < 0.05$, Figure 16); this effect was also reversed by 100nM recombinant agouti protein (Figure 16), demonstrating that the anti-lipolytic effect of agouti is distal to the ACTH receptor.

We have previously shown recombinant agouti protein to increase intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) in several cell types, including human adipocytes (11). To determine the role of $[\text{Ca}^{2+}]_i$ in the anti-lipolytic effect of agouti, human adipocytes were treated with KCl or arginine vasopressin (AVP) to stimulate voltage and receptor-operated Ca^{2+} influx, respectively. As shown in Figure 17, depolarizing human adipocytes with 25 mM and 40 mM KCl for 1 hr inhibited 1 μ M forskolin-induced lipolysis by 42 % and 50 %, respectively ($P < 0.05$). Similarly, short-term treatment with 1 nM AVP caused a 56 % inhibition in forskolin-induced lipolysis in human adipocytes ($P < 0.01$, Figure 18). This suggests that increasing $[\text{Ca}^{2+}]_i$ by either KCl or AVP mimicked the anti-lipolytic effect of agouti.

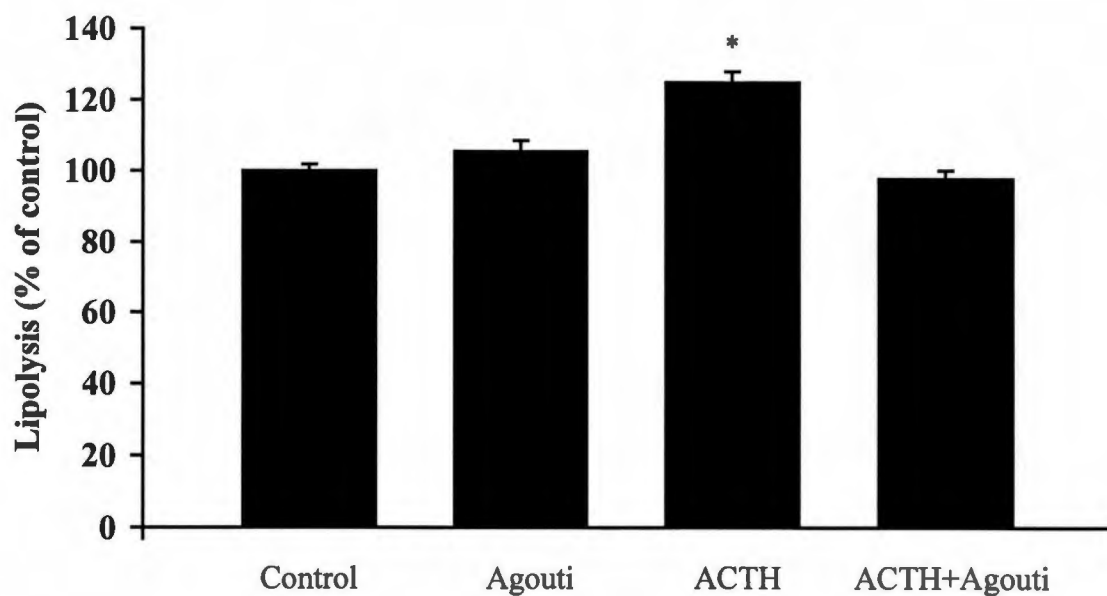


Figure 15. Effect of 1-hr agouti treatment on ACTH-induced lipolysis in human adipocytes. Primary cultured human adipocytes were incubated with 100 nM recombinant agouti protein, 50 nM ACTH or both for 1 hour. Lipolysis was determined as described in Materials and Methods. Data are expressed as mean $\pm$ SEM for 8 experiments. * $P < 0.05$ vs. other treatments.

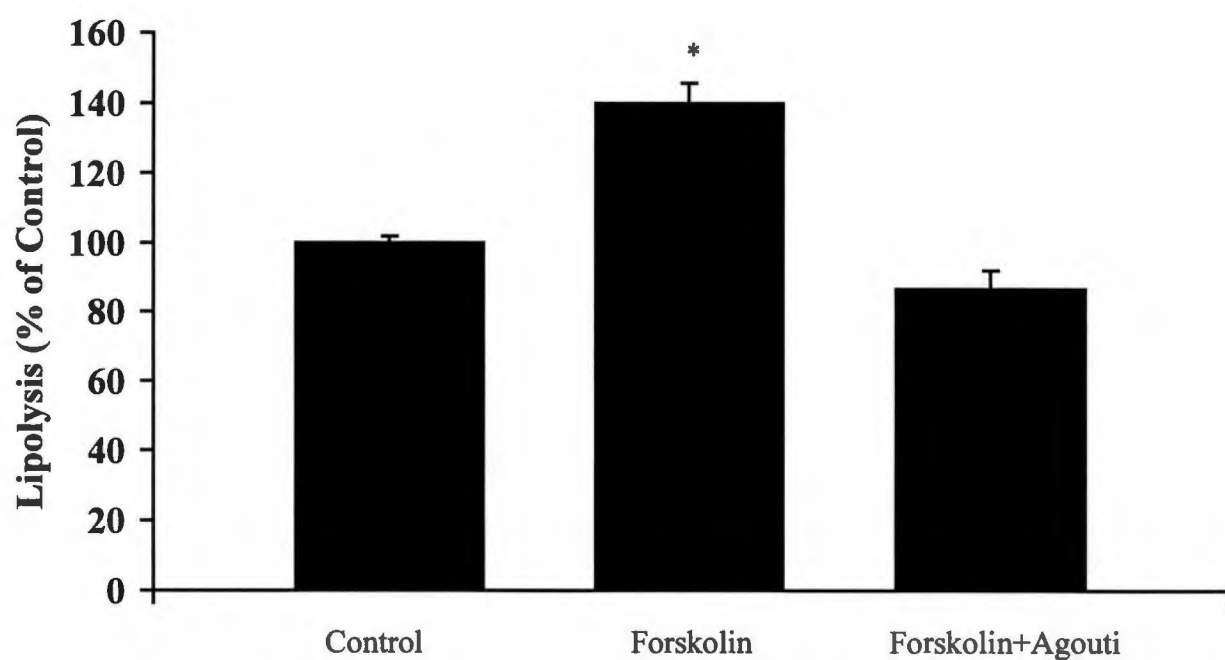


Figure 16. Effect of 1-hr agouti treatment on forskolin-induced lipolysis in human adipocytes. Human adipocytes were incubated with 1 μ M forskolin or 1 μ M forskolin plus 100 nM recombinant agouti protein for 1 hour. Lipolysis was determined as described in Materials and Methods. Data are presented as mean $\pm$ SEM for 4 experiments. * $P < 0.005$ vs. other treatments.

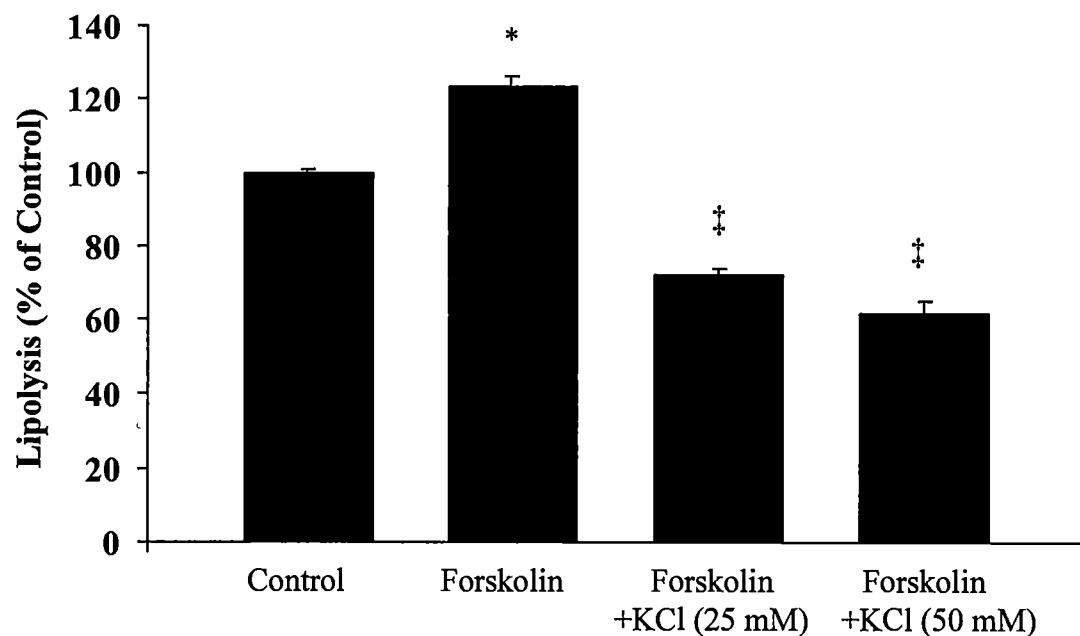


Figure 17. Effect of 1-hr KCl treatment on forskolin-induced lipolysis in human adipocytes. Human adipocytes were treated with 1 μ M forskolin alone or 1 μ M forskolin plus either 25 mM KCl or 40 mM KCl for 1 hour. Lipolysis was determined as described in Materials and Methods. Data are presented as mean $\pm$ SEM for 4 experiments. * $p < 0.05$ vs Control. ‡ $p < 0.05$ vs Forskolin or Control

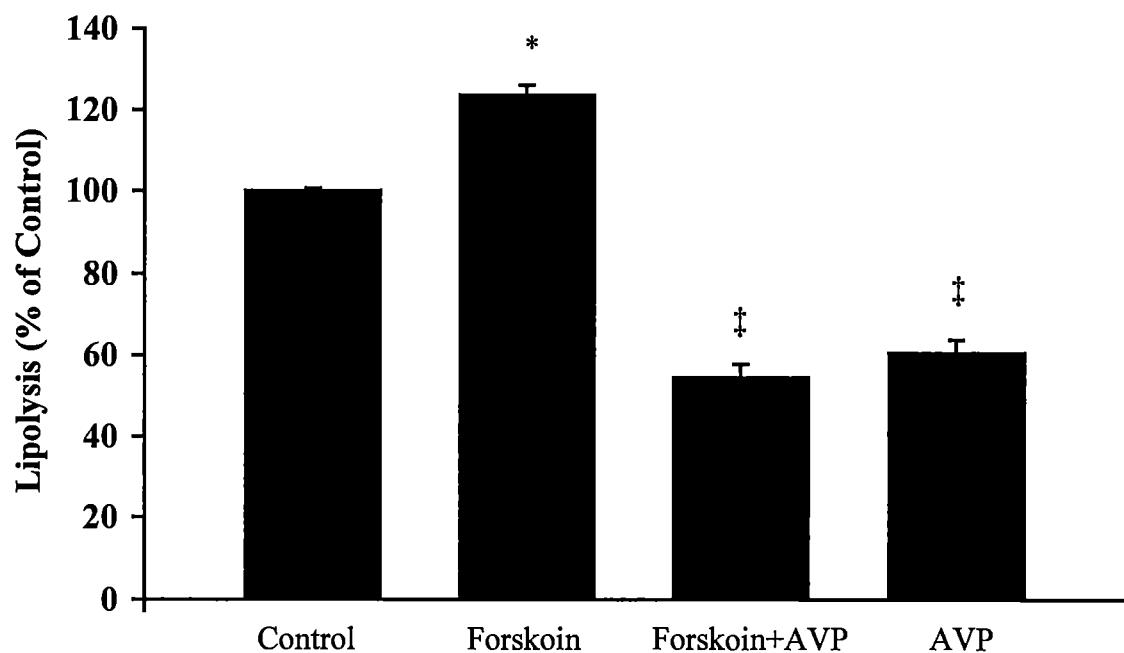


Figure 18. Effect of 1-hr AVP treatment on basal and forskolin-induced lipolysis in human adipocytes. Human adipocytes were incubated with 1 μ M forskolin, 1 nM AVP or both for 1 hour. Lipolysis was determined as described in Materials and Methods. Data are presented as mean $\pm$ SEM for 4 experiments. * $p < 0.05$ vs Control. ‡ $p < 0.01$ vs Forskolin or Control

Although short-term exposure of human adipocytes to 100nM recombinant agouti did not affect basal lipolysis, acute treatment of adipocytes with AVP or KCl did inhibit basal lipolysis as shown in Figure 18 and 19, respectively. To further investigate whether agouti affects basal lipolysis in the absence of lipolytic agents, we incubated human adipocytes with 100 nM agouti for up to 24 hours. As shown in Figure 20, 24-hr treatment of agouti caused a 60 % decrease in lipolysis compared to control ($P<0.0001$). This effect was blocked by 30 μ M nitrendipine (Figure 20) and mimicked by 10 mM KCl (Figure 21).

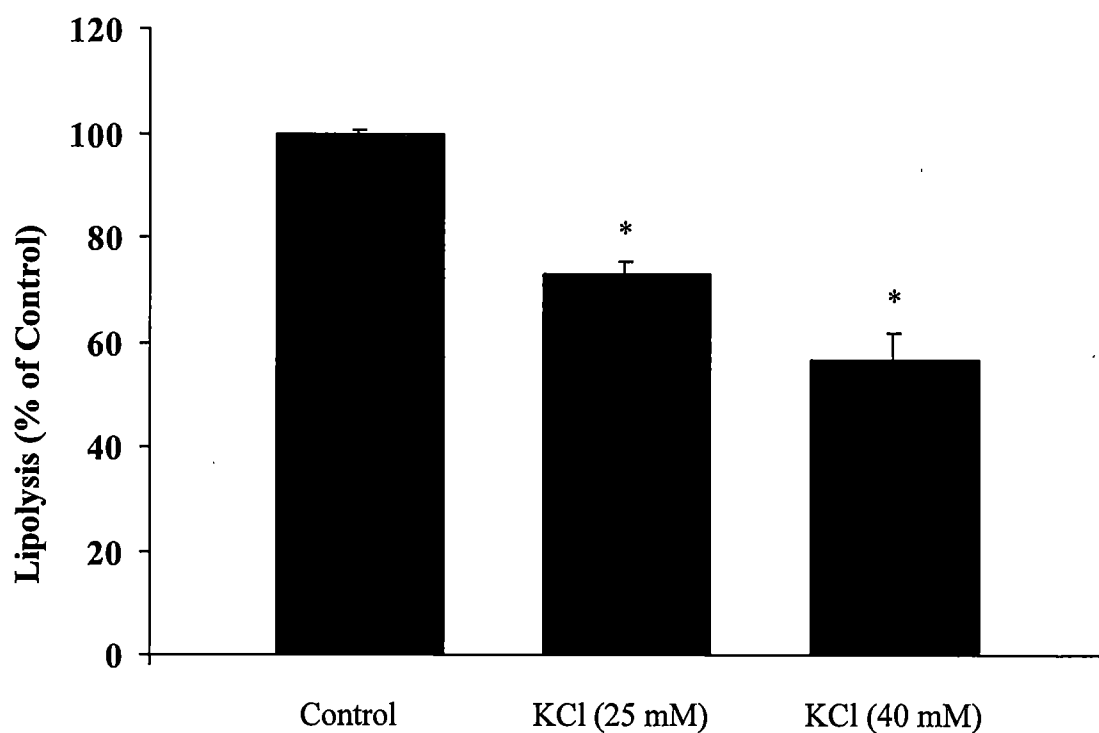


Figure 19. Effect of 1-hr KCl treatment on basal lipolysis in human adipocytes. Human adipocytes were treated with 25 mM KCl or 40 mM KCl for 1 hour. Lipolysis was determined as described in Materials and Methods. Data are presented as mean $\pm$ SEM for 4 experiments. * $p < 0.05$ vs. Control.

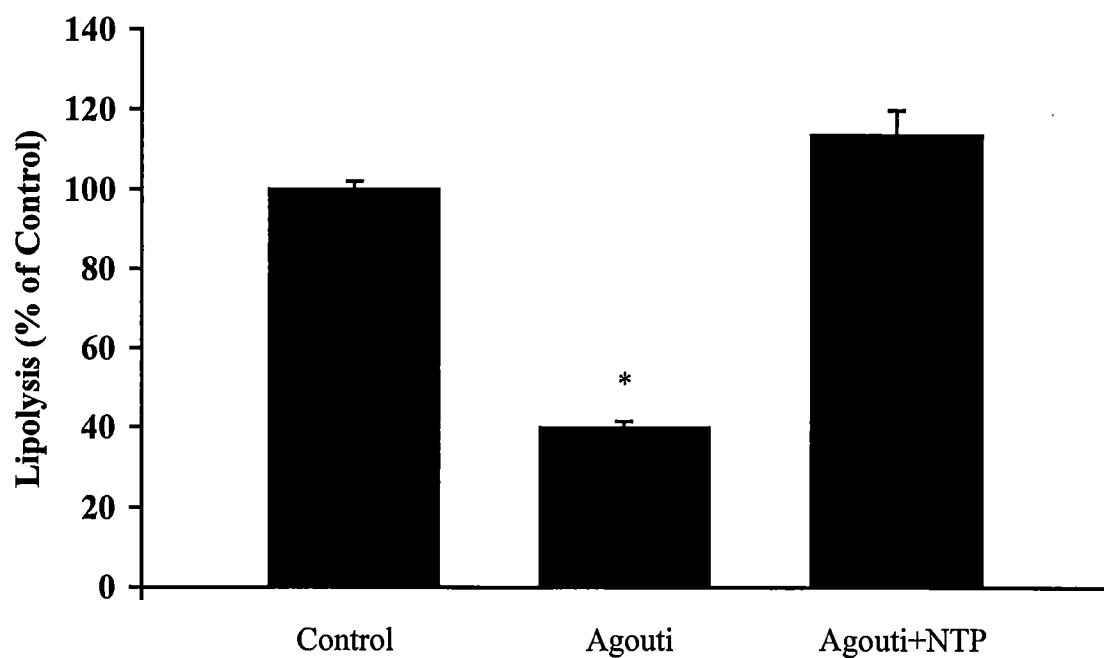


Figure 20. Effect of 24-hr agouti treatment on basal lipolysis in human adipocytes.

Human adipocytes were incubated with either 100 nM agouti or 100 nM agouti plus 30 μ M nitrendipine for 24 hours. Lipolysis was determined as described in Materials and Methods. Data are presented as mean $\pm$ SEM for 4 experiments. * $p < 0.0001$ vs. Control.

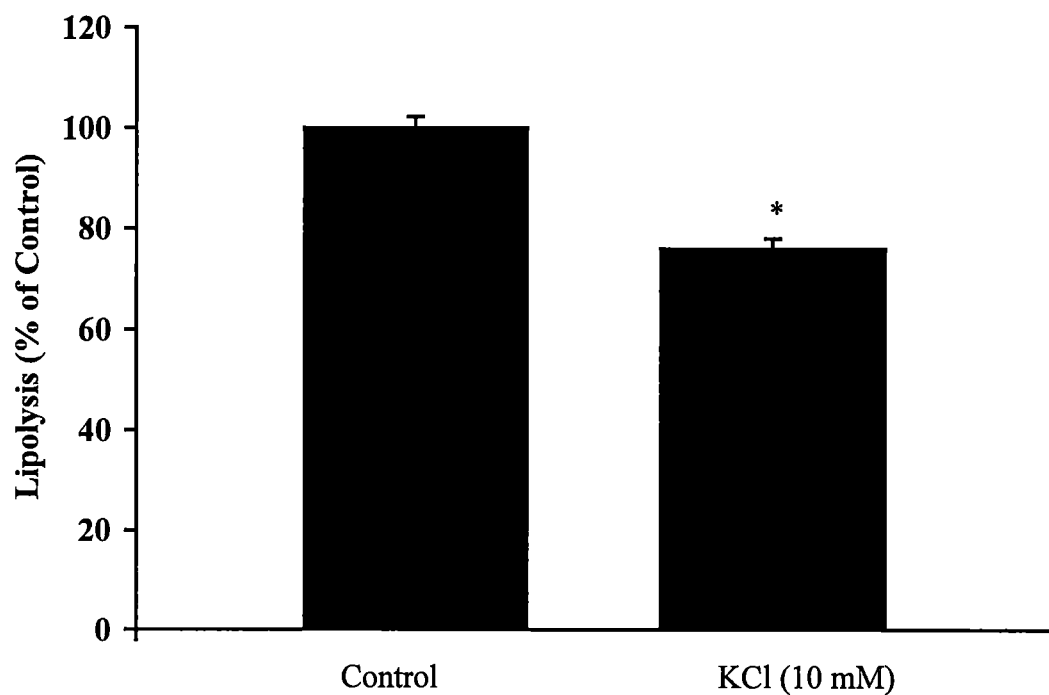


Figure 21. Effect of 24-hr KCl treatment on basal lipolysis in human adipocytes. Human adipocytes were incubated with or without 10 mM KCl for 24 hours. Lipolysis was determined as described in Materials and Methods. Data are presented as mean $\pm$ SEM for 4 experiments. * $p < 0.005$ vs. Control.

V. DISCUSSION

Previous studies of isolated adipose tissue *in vitro* demonstrated that the basal lipolytic rate of adipose tissue from yellow *agouti* mice was about 50% of that of wild type littermates (20-22). The response of this obese adipose tissue to epinephrine (21), theophylline (21) and the β agonists LY79771 (22) and LY104119 (20) was also lower than normal. It was proposed that this may not result from the direct action of *agouti* protein, as decreased lipolysis was also observed in other obese models, including *ob/ob* and *db/db* mice compared to controls (3).

However, our data demonstrated that recombinant *agouti* protein directly inhibited basal and agonist-stimulated lipolysis in primary cultured human adipocytes. We propose that this may represent a coordinated regulation of lipid metabolism in adipose tissue by *agouti*, as *agouti* inhibits lipolysis at the same dosage at which it stimulates lipogenesis (13). This coordinated process may be responsible for the accumulation of triglyceride in adipose tissue and may thereby contribute to the development of obesity. As the human homologue of *agouti* is expressed in adipose tissue (16), *agouti* may exert its action on adipocytes via a paracrine mechanism, similar to its action in pigment regulation within the hair bulb.

Agouti regulation of coat color is exerted through competitive antagonism of α -MSH binding to the melanocortin receptor (MCR-1; 7). *Agouti* is also a potent antagonist of other melanocortin receptors (8). Since MCR-2 (the ACTH receptor) and MCR-5 are expressed in adipocytes (24), and ACTH is a potent lipolytic hormone (25), it is possible that *agouti*'s anti-lipolytic effect may similarly involve competitive antagonism of this receptor. However, our data show that, although *agouti* blocked ACTH-induced lipolysis

in primary cultured human adipocytes, it also blocked forskolin-induced lipolysis. This demonstrates that the anti-lipolytic effect of agouti is distal to the ACTH receptor. This concept is further supported by agouti inhibition of basal lipolysis in the 24 hr treatment, even in the absence of ACTH.

We have found agouti to regulate Ca^{2+} signaling in several cell types (11), including adipocytes. Recent data demonstrated that increasing $[\text{Ca}^{2+}]_i$ by AVP and epidermal growth factor (EGF) in rat adipocytes inhibits agonists-induced lipolysis in a dose-dependent manner (24). Our data show that increasing Ca^{2+} -influx into adipocytes through either voltage- or receptor-operated Ca^{2+} -channels, by KCl or AVP, respectively, also inhibited forskolin-induced lipolysis. KCl (10mM) also inhibited basal lipolysis in the long-term (24 hr) treatment. Further, the inhibitory effect of agouti on basal and forskolin-induced lipolysis was blocked by the Ca^{2+} -channel antagonist nitrendipine. These data demonstrate that agouti's anti-lipolytic effect is mediated by Ca^{2+} signaling.

$[\text{Ca}^{2+}]_i$ may play an important role in the metabolic disorder of obesity and insulin resistance (17-19). Obese patients exhibit elevated basal $[\text{Ca}^{2+}]_i$ in adipocytes (19). An optimal range of $[\text{Ca}^{2+}]_i$ has been demonstrated for maximizing insulin-stimulated glucose transport, with elevation beyond this optimal range causing diminished cellular responsiveness to insulin (18). Similarly, although Ca^{2+} is known to be necessary for ACTH-induced lipolysis in adipocytes (30,31), our data demonstrated that elevating $[\text{Ca}^{2+}]_i$ results in reduced lipolysis. This suggests that an optimal range of $[\text{Ca}^{2+}]_i$ may also exist for maximizing agonist-stimulated lipolysis.

Although we have demonstrated that recombinant agouti protein exerted anti-lipolytic effect in human adipocytes, the target of agouti inhibition of lipolysis is not

clear. Since agouti inhibited both ACTH- and forskolin-induced lipolysis, it is possible that agouti may cause an impairment in cAMP signaling beyond adenylate cyclase. Yen et al (22) demonstrated that while adipose tissue from yellow agouti mice exhibited lower lipolytic rate in response to epinephrine and theophylline, the response to dibutyryl cAMP, a permeable cAMP analogue, was normal. This indicates that agouti may inhibit the generation of cAMP. The sustained inhibition of lipolysis seen in long-term treated human adipocytes may be due to prolonged impairment in cAMP production. Alternatively, we have demonstrated that there is an agouti response element in the fatty acid synthase (FAS) promoter (32). It is possible that hormone sensitive lipase expression may be similarly modulated by agouti, although this possibility has not yet been investigated.

In summary, the present study shows that recombinant agouti protein exhibits potent anti-lipolytic effect via a Ca^{2+} -dependent mechanism in primary cultured human adipocytes. This appears to be a direct effect that is not mediated by MCR antagonism. This inhibition of lipolysis, combined with agouti-induced lipogenesis, represents a coordinate control of adipocyte lipid metabolism which may contribute to agouti-induce obesity.

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PART 6

MECHANISM OF INTRACELLULAR CALCIUM ($[Ca^{2+}]_i$) INHIBITION OF

LIPOLYSIS IN HUMAN ADIPOCYTES¹

¹This manuscript has been submitted for publication in similar form in: J. Clin. Endocrinol. Metab.

I. ABSTRACT

We recently reported that increasing $[Ca^{2+}]_i$ exerts an anti-lipolytic effect via a post-receptor mechanism. In the present study, we investigated the mechanisms responsible for this anti-lipolytic effect of $[Ca^{2+}]_i$ in human adipocytes. Depolarizing adipocytes with 100 mM KCl inhibited lipolysis induced by activation of β -adrenergic receptor, inhibition of the selective A1 adenosine receptor, activation of adenylate cyclase, inhibition of phosphodiesterase (PDE), and a cell permeable cAMP analogue that activates protein kinase A (PKA), although with different potencies. However, lipolysis stimulated by the non-hydrolyzable cAMP analogue, dibutyryl cAMP was not affected, indicating that the anti-lipolytic effect of KCl may be mediated by the activation of adipocyte phosphodiesterase. Consistent with this, isobutyl methylxanthine (IBMX) completely reversed KCl inhibition of isoproterenol stimulated lipolysis. In addition, KCl inhibition of isoproterenol-stimulated lipolysis was reversed by the selective PDE 3B inhibitor, cilostamide, but not by selective PDE 1 and 4 inhibitors, suggesting that KCl may exert its anti-lipolytic effect via activation of PDE 3B, the same PDE isoform that mediates insulin's anti-lipolytic effect. However, the anti-lipolytic effects of insulin and KCl were additive. In addition, phosphatidylinositol-3 (PI-3) kinase inhibition with wortmannin completely prevented insulin's anti-lipolytic effect, but only minimally blocked KCl inhibited lipolysis. These data suggest that KCl and insulin may activate PDE 3B via different mechanisms. Further, the anti-lipolytic effect of KCl is not affected by inhibitors of calmodulin, Ca^{2+} /calmodulin dependent kinase, protein phosphatase 2B (PP2B, calcinurin) and protein kinase C (PKC). However, KCl's anti-lipolytic effect was abolished by the protein phosphatase 1 and 2A (PP1 and PP2A) inhibitor, okadaic acid.

Finally, KCl treatment significantly inhibited forskolin-induced phosphorylation of hormone sensitive lipase, the rate-limiting enzyme in lipolysis, in ^{32}P -labeled human adipocytes. We conclude that increasing $[\text{Ca}^{2+}]_i$ exerts an anti-lipolytic effect mainly by activation of PDE, thereby resulting in a net decrease in HSL phosphorylation and, consequently, inhibition of lipolysis.

II. INTRODUCTION

Previous data from our laboratory demonstrates that agouti protein, a murine obesity gene product, inhibits human adipocyte lipolysis via a Ca^{2+} -dependent mechanism (1), resulting in increased adipocyte lipid storage. In addition, several studies have reported direct anti-lipolytic effects of increasing intracellular calcium ($[\text{Ca}^{2+}]_i$) under different conditions (2-5). However, the mechanisms responsible for this anti-lipolytic effect of $[\text{Ca}^{2+}]_i$ is unclear. Although altered interaction between G_i and adenylate cyclase has been reported in one study, others suggest that alteration in adipocyte phosphodiesterase activity may be responsible in the anti-lipolytic effect of increasing cellular Ca^{2+} concentrations (3-6).

Adipocyte lipolysis is under acute hormonal regulation (reviewed in 7,8). Lipolytic hormones, such as catecholamines, act through β -adrenergic receptors, resulting in increases in cAMP, which then activates cAMP dependent protein kinase A (PKA). The activation of PKA in turn phosphorylates and activates hormone-sensitive lipase (HSL), a key enzyme in lipolysis. Insulin, in contrast, acting through the insulin receptor and downstream phosphatidylinositol-3 (PI-3) kinase and possibly other downstream kinases, phosphorylates and activates a phosphodiesterase kinase, which is now believed to be protein kinase B (PKB) (9). PKB then phosphorylates and activates phosphodiesterase 3B (PDE 3B), a major PDE 3 isoform expressed in adipose tissue, which catalyzes the degradation of cAMP, leading to a decrease in cAMP and PKA activity, and inhibition of lipolysis (reviewed in 7,8).

HSL is primarily regulated by reversible phosphorylation. Phosphorylation of the regulatory site upon lipolytic stimulation results in activation of HSL (10-12).

Phosphorylation of another serine residue, the basal site, mainly occurs in resting cells and has been shown to exert an anti-lipolytic effect so that its phosphorylation prevents the phosphorylation of the regulatory site (13,14). Recent evidence suggests that additional serine residues phosphorylated during HSL activation are crucial in governing HSL activity (15).

The present study selectively evaluated the effects of $[Ca^{2+}]_i$ on each of these steps to determine the mechanisms responsible for the anti-lipolytic effect of $[Ca^{2+}]_i$ in human adipocytes. We report here that depolarizing human adipocytes with 100 mM KCl exerts an anti-lipolytic effect primarily via activation of PDE 3B. This results in a net decrease in HSL phosphorylation and, consequently, inhibition of lipolysis.

III. MATERIALS AND METHODS

A. Cell Culture

Human adipocytes were either obtained from Zen-Bio (Research Triangle, NC) or differentiated from human adipose tissue stromal vascular cells (Zen-Bio, Research Triangle, NC) in our laboratory. To differentiate into mature adipocytes, pre-adipocytes were plated at a density of 30,000 cells/cm² with pre-adipocyte medium [Dulbecco's Modified Eagle Medium (DMEM)/Ham's F-10 Nutrient Broth (Ham's F-10), 1:1 (v/v) containing 10 % fetal calf serum, 15 mmol/L 4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic acid (HEPES) and antibiotics]. Cells were then put into differentiation medium [DMEM/Ham's F-10, 1:1 (v/v) containing 10 % fetal bovine serum, 15 mmol/L HEPES, 33 µmol/L biotin, 17 µmol/L pantothenate, 100 nmol/L insulin, 1 µmol/L dexamethasone, 0.25 mmol/L isobutyl methylxanthine (IBMX), 1 µmol/L BRL49653 and antibiotics]. After three days in differentiation cocktail, cells were maintained in adipocyte medium [DMEM/Ham's F-10, 1:1 (v/v) containing 3 % fetal bovine serum, 15 mmol/L HEPES, 33 µmol/L biotin, 17 µmol/L pantothenate, 100 nmol/L insulin, 1 µmol/L dexamethasone and antibiotics] until fully differentiated.

B. [Ca²⁺]_i measurement

Adipocyte [Ca²⁺]_i was determined using a fura-2 dual wave length fluorescence imaging system (Intracellular Imaging Inc., Cincinnati, OH). Human pre-adipocytes were plated in 35 mm glass plates (Mat Tek Co., Ashland, MA) and differentiated as described above. Prior to [Ca²⁺]_i measurement, cells were maintained serum-free overnight and then washed with HEPES balanced salt solution (HBSS, containing components in mM: NaCl 138, CaCl₂ 1.8, MgSO₄ 0.8, NaH₂PO₄ 0.9, NaHCO₃ 4, glucose 5, glutamine 6,

HEPES 20 and bovine serum albumin 1%). Cells were then loaded with 10 μ M fura-2 acetoxymethyl ester (fura-2 AM) and incubated at 37°C in the dark for 2 hrs. To remove extracellular dye, cells were rinsed with HBSS and kept at room temperature for an additional 1 hr for complete hydrolysis of cytoplasmic fura-2 AM. Cells were then mounted on the stage of Nikon TMS-F fluorescence inverted microscope with a Cohu 4915 CCD camera. Fluorescent images were captured alternatively at excitation wavelength of 340 and 380 nm with an emission wavelength of 510 nm. Images were analyzed with InCyt Im2 version 4.62 imaging software (Intracellular Imaging Inc., Cincinnati, OH). $[Ca^{2+}]_i$ was calculated using a ratio equation as described previously (16). Each analysis evaluated responses of 6-10 cells. Images were calibrated using fura-2 calcium imaging calibration kit (Molecular Probes, Eugene, OR) to create a calibration curve in solution. Cellular calibration was accomplished using digitonin (25 μ M) and Tris (pH 8.7)/ethylene glyco-bis (β -aminoethyl ether) N, N, N', N'-tetraacetic acid (EGTA, 100 mM) to measure maximal and minimal intracellular Ca^{2+} levels (16).

C. Lipolysis experiments

Differentiated human adipocytes were incubated in serum-free medium overnight prior to lipolysis experiments. Cells were subjected to the treatments described in each figure legend and table in Krebs-Ringer buffer containing 25 mM HEPES and 1.5 % bovine serum albumin (KRH buffer) for 4 hrs. When 100 mM KCl was used in the treatment, equimolar amounts of NaCl were removed to maintain osmolarity. Lipolysis was determined as glycerol release into the culture medium by an enzymatic fluorometric method (17) using a microplate fluorometer (Packard Instrument Company, Inc., Downers Grove, IL).

Total DNA was measured by CyQUANT cell proliferation assay kit according to manufacturer's instruction (Molecular Probes, Eugene, OR).

Cell viability was determined by Trypan Blue exclusion examination and medium lactate dehydrogenase (LDH) activity measurement with a LDH based in vitro toxicology assay kit (Sigma, St. Louis, MO).

D. cAMP measurement

To measure cAMP levels, human adipocytes were incubated in serum-free medium overnight and subjected to isoproterenol stimulation either with or without KCl or insulin in KRH buffer for 10 min. Cells were immediately lysed, and cellular cAMP levels were measured using a cAMP ^{125}I scintillation proximity assay (SPA) system (Amersham Pharmacia Biotech, Piscataway, NJ).

E. ^{32}P -labeling of human adipocytes and analysis of adipocyte phosphoproteins by sodium dodecyl sulfate (SDS) – polyacrylamide gel electrophoresis (PAGE)

The analysis of ^{32}P -labeled human adipocyte phosphoprotein was conducted according to Holm et al., Dergerman et al. and Castan et al (8, 18, 19). Human adipocytes were incubated in serum-free medium overnight and prelabeled for 90 min at 37°C with 0.4-0.8 mCi/ml ^{32}P -orthophosphate (ICN,) in KRB buffer containing 0.3 mM KH_2PO_4 . After labeling, cells were subjected to stimulation by isoproterenol either with or without KCl or insulin for 10 min. The incubations were terminated by the addition of homogenization buffer [50 mM Tris-HCl (pH 7.4), 250 mM sucrose, 1 mM EDTA, 1 mM dithioerythritol, 1 % (v/v) Triton X-100, 10 % protease cocktail (Sigma, St. Louis, MO), 1 % each of serine phosphatase and tyrosine phosphatase inhibitor cocktail (Sigma, St. Louis, MO)]. Cells were sonicated on ice for 10 sec and incubated on ice for 1 hr to

solubilize all proteins. Insoluble material was removed by centrifugation at 12,500 x g at 4 °C for 10 min. Protein from equal number of cells (measured as equal amount of DNA) was boiled in Laemmli sample buffer (20) and subject to 10 % SDS-PAGE (20). After staining with Coomassie Blue, gels were dried and adipocyte phosphoproteins were visualized by autoradiography.

F. Immunoprecipitation

Immunoprecipitation of adipocyte phosphoproteins was performed according to Castan et al. (19). Briefly, human adipocytes were prelabeled with ^{32}P -orthophosphate and treated as described above. Adipocyte lysates were then prepared as described above, and equal amounts of lysates were incubated with an anti-HSL antibody (21) at 4 °C overnight on an orbital shaker. Lysates were then incubated with Protein A-Sepharose 4B-CL (Sigma, St. Louis, MO) for 4 hrs at 4 °C. The immunocomplexe was washed 5 times with PBS containing 0.1 % (w/v) N-laurylsarcosin, boiled in Laemmli sample buffer. The phosphorylated HSL was analyzed as above.

Statistical analysis

All data are expressed as means $\pm$ SEM. Data were analyzed using the procedures of SPSS Inc. (Chicago, IL). Difference of lipolysis among treatment groups were compared using one-way analysis of variance (One-Way ANOVA). A p-value < 0.05 is considered significant.

IV. RESULTS

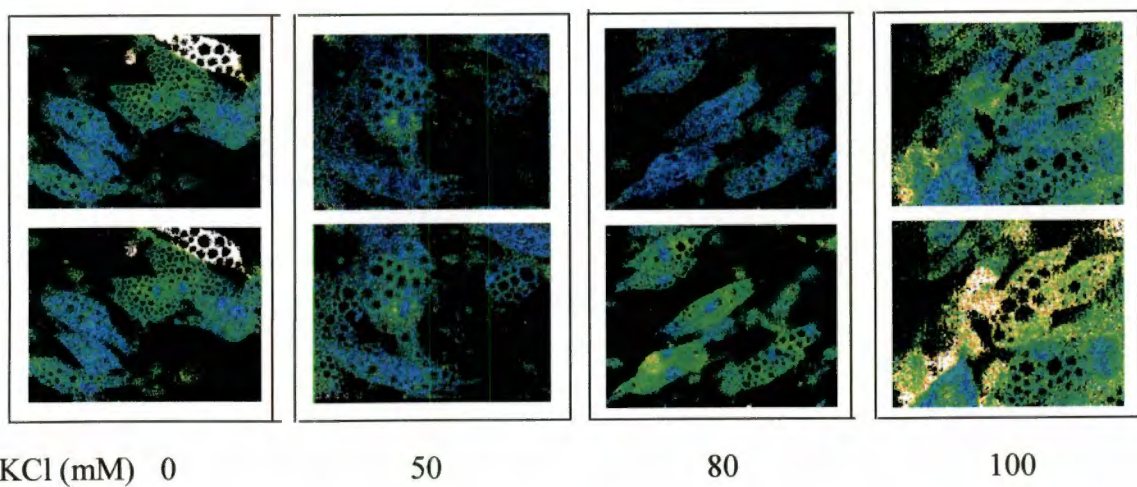
We utilized KCl as a depolarizing agent to stimulate $[Ca^{2+}]_i$ in human adipocytes. We first studied the effect of KCl on human adipocyte $[Ca^{2+}]_i$. Figure 22A shows fluorescent imaging of adipocyte $[Ca^{2+}]_i$ in pseudocolor, which changes from blue/green in baseline to green/yellow/white upon addition of KCl, indicating an increase in $[Ca^{2+}]_i$. Quantitation of this response demonstrated a dose-dependent increase in $[Ca^{2+}]_i$ in these cells, with 17.86 ± 1.65 nM, 87 ± 5.79 nM and 158.71 ± 8.55 nM increases over baseline in response to 50, 80 and 100 mM KCl stimulation, respectively ($p < 0.05$, Figure 22B).

KCl treatment also significantly inhibited 0.1 μ M forskolin-stimulated lipolysis in these adipocytes, with the greatest effect at 100 mM (Figure 22C). During these experiments, cell viability was verified using both Trypan Blue exclusion examination and medium lactate dehydrogenase activity measurement.

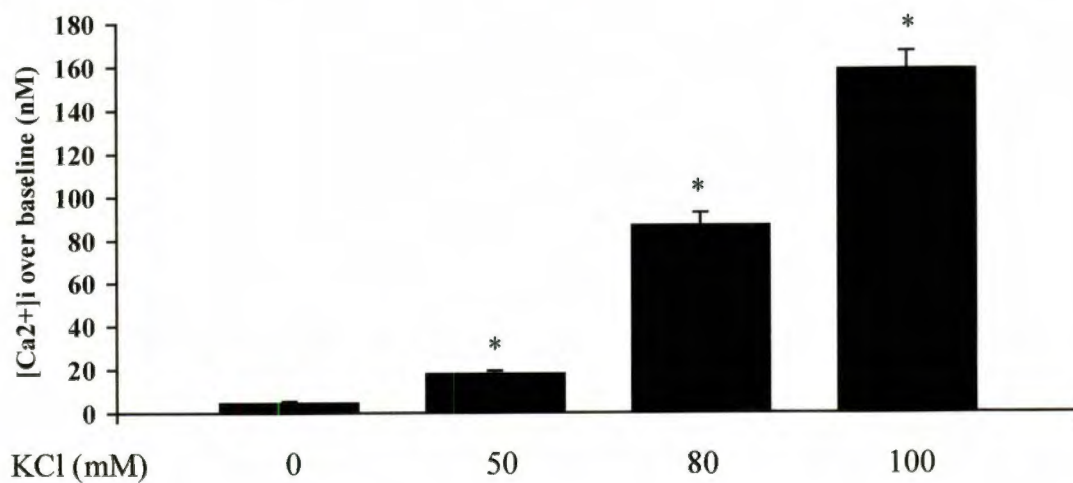
We next tested the ability of 100 mM KCl to inhibit lipolytic responses to the following agonists: isoproterenol, a β -adrenergic receptor agonist; 8-cyclopentyl-1,3-dipropyl xanthine (DPCPX), a selective A1 adenosine receptor antagonist; forskolin, an adenylate cyclase activator; isobutyl methyl xanthine (IBMX), a phosphodiesterase inhibitor; 8-bromo-cAMP and dibutyryl cAMP, hydrolyzable and non-hydrolyzable cAMP analogues, respectively, which activate protein kinase A. Figure 23 demonstrates that KCl treatment inhibited all except dibutyryl cAMP induced lipolysis in human adipocytes. 8-bromo-cAMP is susceptible to hydrolysis by phosphodiesterase whereas dibutyryl cAMP is resistant to hydrolysis by this enzyme. In addition, insulin, which inhibits adipocyte lipolysis by activation of phosphodiesterase, also inhibited 8-bromo-cAMP induced lipolysis by 90 %; however, it is unable to inhibit dibutyryl cAMP

Figure 22. Effect of KCl on human adipocyte $[Ca^{2+}]_i$ (A and B) and forskolin-stimulated lipolysis (C). A. Human pre-adipocytes were plated and differentiated into mature adipocytes. Adipocytes were loaded with fura-2/AM and $[Ca^{2+}]_i$ was measured using a fura-2 dual wavelength imaging system. The upper and lower panels represent fluorescent image of adipocyte $[Ca^{2+}]_i$ before and after the addition of vehicle (H_2O , Control group), and 50, 80 and 100 mM KCl, respectively. B. Quantification of KCl-stimulated increase in $[Ca^{2+}]_i$. * $p < 0.05$ vs. Control. C. Human adipocytes were treated with 0.1 μM forskolin and the indicated concentrations of KCl for 4 hrs. Data are expressed as mean $\pm$ SEM for 6 experiments. * $p < 0.05$ vs. no KCl treatment.

A



B



C

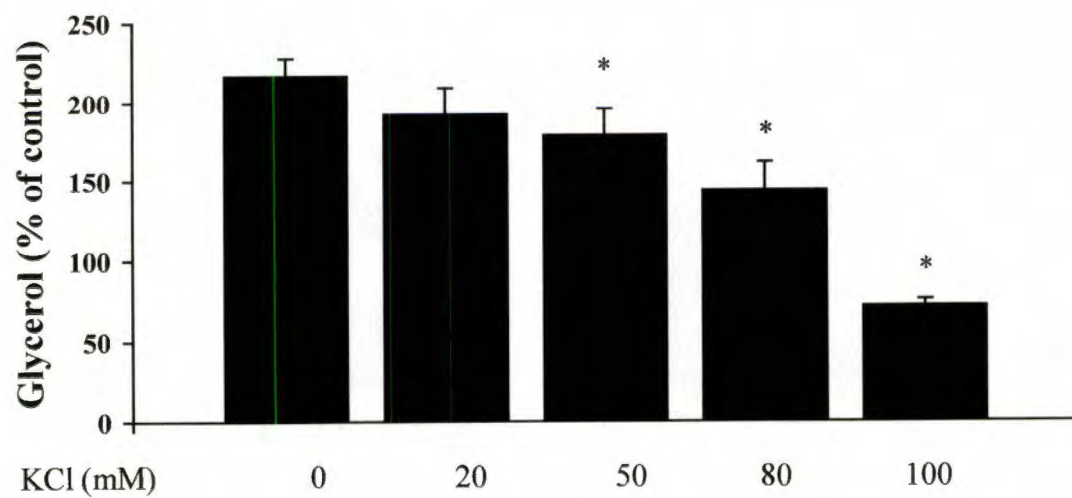
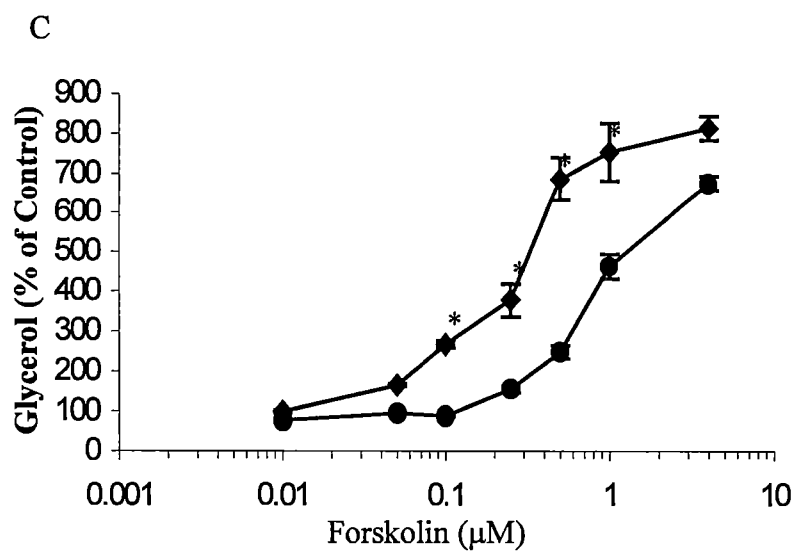
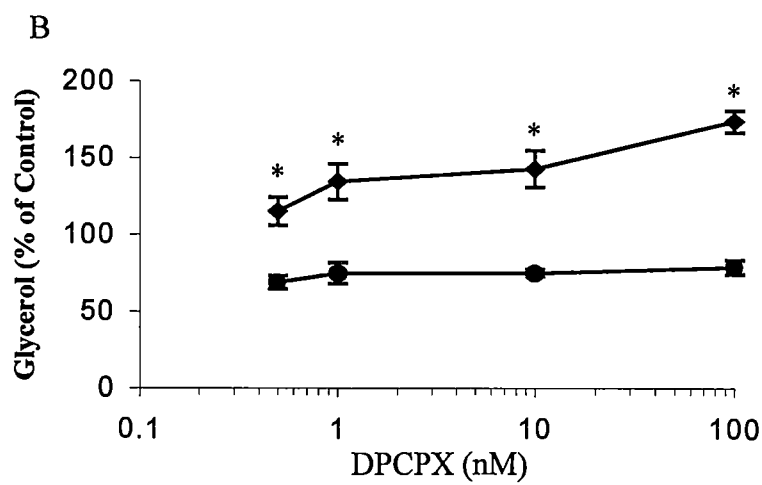
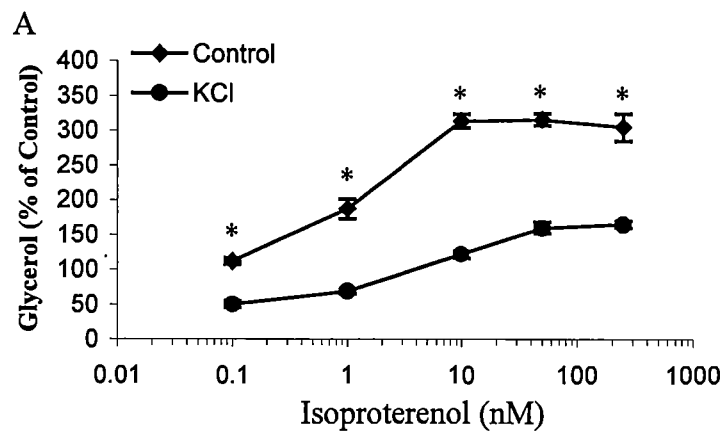
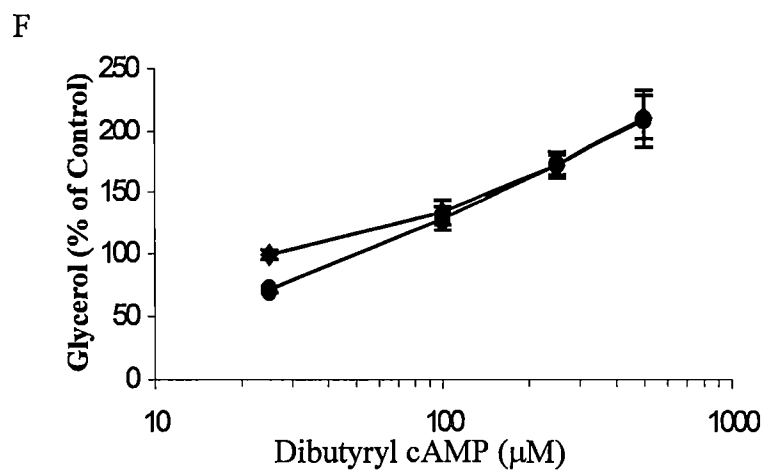
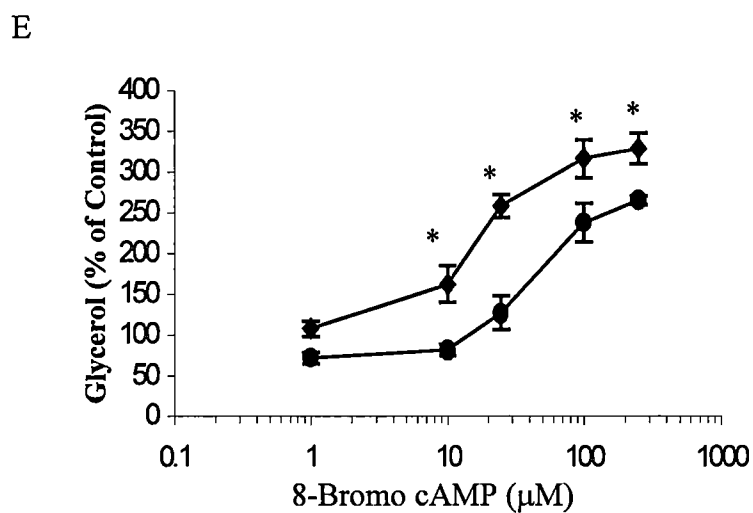
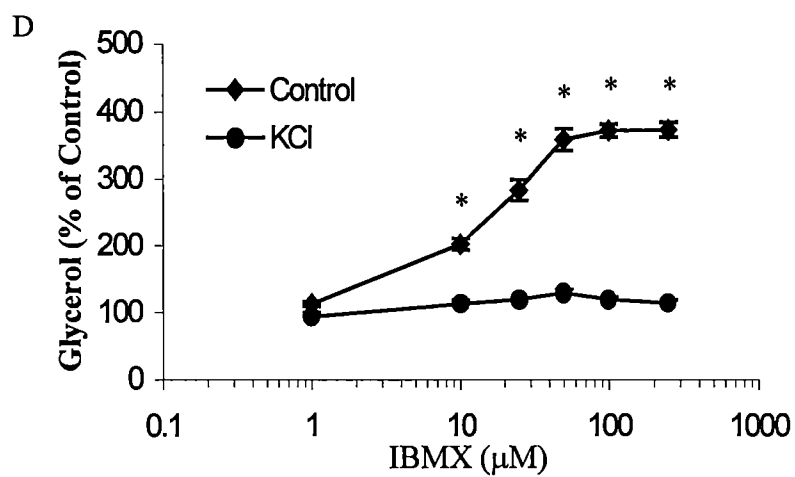


Figure 23. Effect of 100 mM KCl on lipolysis in human adipocytes stimulated by various agonists. Human adipocytes were treated with KCl and isoproterenol (A), DPCPX (B), forskolin (C), IBMX (D), 8-bromo-cAMP (E) and dibutyryl cAMP (F) for 4 hrs. Data are expressed as mean $\pm$ SEM for 6 experiments. * $p < 0.05$ vs. KCl treatment.





induced lipolysis (22, 23). Our observation that KCl inhibited 8-bromo cAMP- stimulated lipolysis, while it was unable to inhibit dibutyryl cAMP- induced lipolysis suggests that KCl may exert its anti-lipolytic effect primarily via activation of phosphodiesterase, similar to insulin. In addition, while KCl completely inhibited isoproterenol, DPCPX and IBMX induced lipolysis, it right-shifted the dose-response curve of forskolin (EC_{50} from 0.17 to 0.60 μ M), although the inhibitory effect is still evident even at a maximum dose of forskolin.

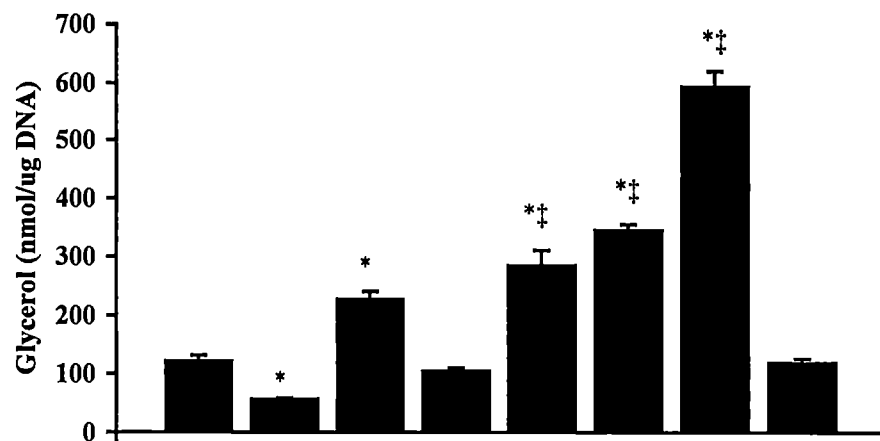
To further investigate the mechanisms of KCl's anti-lipolytic effect, we next studied the ability of different lipolytic agonists to reverse KCl inhibited lipolysis. Figure 24A shows that forskolin, IBMX and dibutyryl cAMP completely reversed KCl's inhibitory effect in human adipocytes treated with isoproterenol. These data, combined with data from Figure 23, further suggest that KCl may exert its anti-lipolytic effect via a step before PKA, i. e. activation of PDE, and, possibly, inhibition of adenylate cyclase.

Xanthine derivatives such as IBMX have been shown to act not only as PDE inhibitors but also as adenosine receptor antagonists (24). The adipocyte A1 adenosine receptor exerts an anti-lipolytic effect by its coupling to G_i , with consequent inhibition of adenylate cyclase (25). In the present study, the selective A1 adenosine receptor antagonist DPCPX alone resulted in a 1.7-fold increase in adipocyte lipolysis (Figure 23B). However, it failed to reverse KCl inhibited lipolysis (Figure 24A), indicating that the A1 adenosine receptor is not involved in KCl's anti-lipolytic effect.

We next investigated the involvement of different PDE isoforms in KCl's anti-lipolytic effect. Using selective inhibitors of different types of PDE, it has been reported that adipocytes possess several PDE isoform activities (26-28). In the present study, we

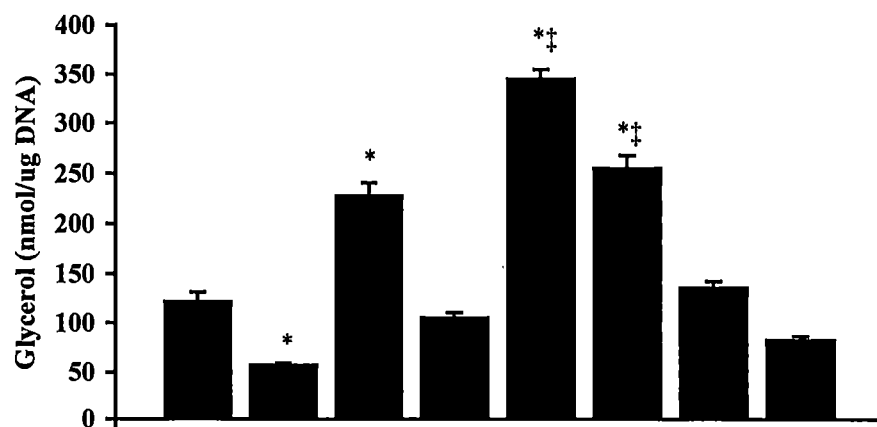
Figure 24. Effects of different agonists on the reversal of KCl inhibition of isoproterenol-stimulated lipolysis. Human adipocytes were treated with isoproterenol, KCl and the indicated agonists for 4 hrs. Data are expressed as mean $\pm$ SEM for 6 replicate experiments. * $p < 0.05$ vs. control. ‡ $p < 0.05$ vs. KCl + Isoproterenol.

A



KCl (100 mM)	-	+	-	+	+	+	+	+
Isoproterenol (5 nM)	-	-	+	+	+	+	+	+
Forskolin (5 μM)	-	-	-	-	+	-	-	-
IBMX (100 μM)	-	-	-	-	-	+	-	-
Dibutyryl cAMP (500 μM)	-	-	-	-	-	-	+	-
DPCPX (100 nM)	-	-	-	-	-	-	-	+

B



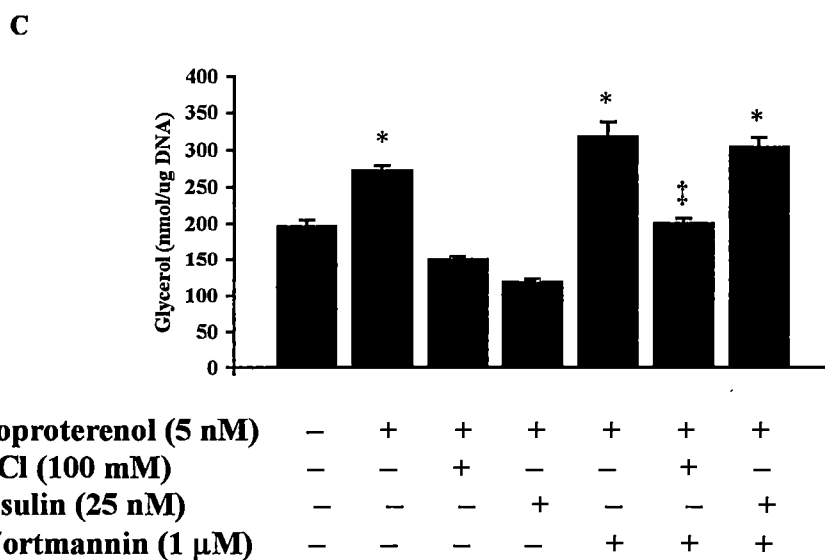
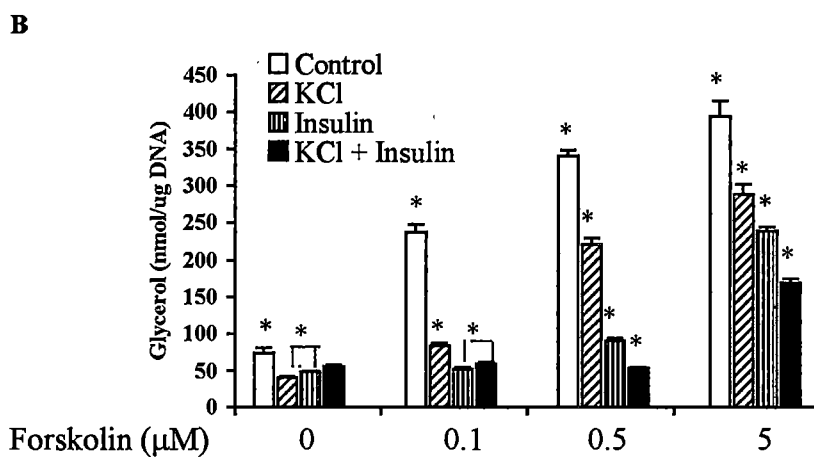
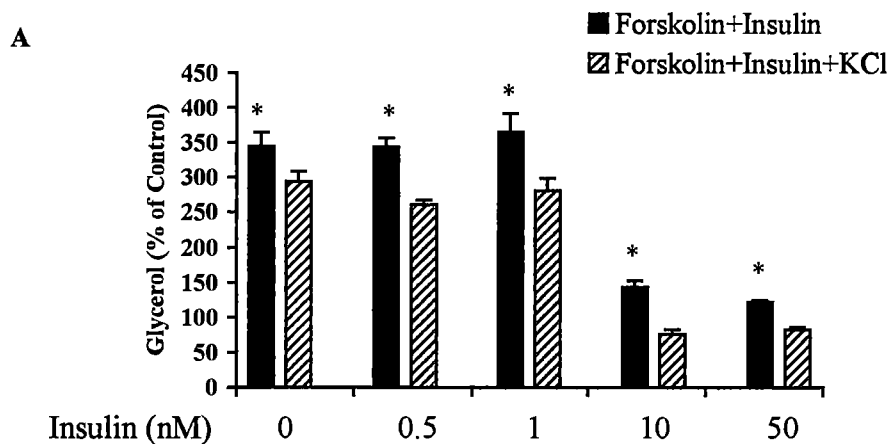
KCl (100 mM)	-	+	-	+	+	+	+	+
Isoproterenol (5 nM)	-	-	+	+	+	+	+	+
IBMX (100 μM)	-	-	-	-	+	-	-	-
Cilostamide (2 μM)	-	-	-	-	-	+	-	-
8-methoxymethyl IBMX(50 μM)	-	-	-	-	-	-	+	-
Rolipram (50 μM)	-	-	-	-	-	-	-	+

found that both the PDE 3B inhibitor cilostamide and the PDE 4 inhibitor 4-[3-(cyclopentyloxy)-4-methoxyphenyl]-2-pyrrolidinone (rolipram) exhibited comparable lipolytic activities in human adipocytes. Cilostamide (2 μ M) caused a 2.7-fold increase in lipolysis (50.19 ± 2.29 vs. 137.17 ± 6.45 nmol/ μ g DNA, $p < 0.05$), while rolipram (50 μ M) caused a 2.2-fold increase (42.92 ± 3.43 vs. 92.42 ± 1.94 nmol/ μ g DNA, $p < 0.05$). Human adipocytes also exhibited less PDE 1 activity, as the PDE 1 inhibitor 8-methoxymethyl IBMX only exerted a 40 % increase in lipolysis (40.98 ± 3.74 vs. 57.48 ± 3.42 nmol/ μ g DNA, $p < 0.05$).

Figure 24B demonstrates that KCl inhibited lipolysis was completely reversed by the non-selective PDE inhibitor, IBMX, and by the selective PDE 3B inhibitor, cilostamide. However, the selective PDE 1 and 4 inhibitors 8-methoxymethyl IBMX and rolipram failed to reverse KCl's anti-lipolytic effect. Cilostamide and its derivative OPC 3911 have been shown to selectively inhibit PDE3B activity and to block the anti-lipolytic effect of insulin in adipocytes (26, 27, 29, 30). These data suggest that KCl's anti-lipolytic effects are mediated by the activation of PDE 3B, the same PDE isoform that mediates insulin's anti-lipolytic effect, but not by the PDE 1 or 4 isoforms.

We next compared the anti-lipolytic effect of KCl to that of insulin. Figure 25A shows that increasing insulin concentrations to 10 nM inhibited 0.1 μ M forskolin stimulated lipolysis. However, this was further inhibited by the addition of KCl, even at maximum insulin concentrations. In Figure 25B, 100 mM KCl and maximum concentration of insulin (25 nM) exerted an additive anti-lipolytic effect in the presence of increasing concentrations of forskolin. These data indicate that the anti-lipolytic effects

Figure 25. The anti-lipolytic effects of KCl and insulin are additive. A. Human adipocytes were treated with 0.1 μ M forskolin, 100 mM KCl and different concentrations of insulin for 4 hrs. Data are expressed as mean $\pm$ SEM for 6 replicate experiments. * $p < 0.05$ vs. KCl treatment. B. Human adipocytes were treated with 25 nM insulin, 100 mM KCl and different concentrations of forskolin for 4 hrs. Data are expressed as mean $\pm$ SEM for 6 experiments. * $p < 0.05$ within each cluster. C. Human adipocytes were treated with isoproterenol in the presence of either KCl or insulin and either with or without wortmannin for 4 hrs. Data are expressed as mean $\pm$ SEM for 6 experiments. * $p < 0.05$ vs. Control. ‡ $p < 0.05$ vs. Isoproterenol + Wortmannin.



of insulin and KCl are additive, suggesting that KCl and insulin may activate PDE 3B via different mechanisms.

Consistent with this, wortmannin, a PI-3 kinase inhibitor, completely reversed insulin's anti-lipolytic effect. However, it only partially blocked KCl's anti-lipolytic effect (Figure 25C). This further suggests that the anti-lipolytic effects of KCl and insulin involve different mechanisms, although both involving PDE 3B activation.

Different calmodulin and Ca^{2+} /calmodulin dependent protein kinase (Ca^{2+} /CaM protein kinase) inhibitors were used to study whether calmodulin and Ca^{2+} /CaM protein kinase are involved in this effect of KCl. The addition of different calmodulin inhibitors trifluoperazine (TFP), N-(6-aminoethyl)-5-chloro-1-naphthalene sulphonamide (W-7) and calmidazolium, as well as the Ca^{2+} /CaM protein kinase inhibitor KN-62, failed to reverse KCl's anti-lipolytic effect even at very high concentrations (Table 2). Instead, with the exception of calmidazolium, high doses of these compounds exerted independent anti-lipolytic effects comparable to that seen with KCl.

We also investigated the possible involvement of protein kinase C (PKC), protein phosphatase 2B (PP2B, calcinurin) and protein phosphatase 1/2A in KCl's anti-lipolytic effect. The addition of the PKC and PP 2B inhibitors chelerythrine chloride and FK 506, respectively, failed to affect KCl's anti-lipolytic effect (Figure 26). However, the PP1/2A inhibitor okadaic acid, at a concentration that inhibits both PP1 and PP2A, completely abolished KCl's anti-lipolytic effect (Figure 26). These data indicate that PP1 and/or PP2A may be involved in KCl's anti-lipolytic effect.

We also studied the effect of KCl on adipocyte cAMP level. In Figure 27, 5 nM isoproterenol treatment resulted in a 5-fold increase in cAMP level (22.75 ± 2.57 vs.

Table 2. Effects of calmodulin and Ca^{2+} /calmodulin dependent protein kinase inhibitors on KCl-inhibited lipolysis

Treatment	Glycerol (nmol/ μg DNA)
Control	12.10 $\pm$ 0.78
Isoproterenol 5 nM	21.28 $\pm$ 1.36 *
Isoproterenol 5 nM + KCl 100mM	13.01 $\pm$ 0.50
+ TFP 10 μM	13.25 $\pm$ 0.64
+TFP 50 μM	10.34 $\pm$ 0.67
+ TFP 100 μM	9.90 $\pm$ 0.78
+ W-7 10 μM	11.54 $\pm$ 0.66
+ W-7 50 μM	10.94 $\pm$ 0.48
+ W-7 100 μM	7.27 $\pm$ 0.79
+ Calmidazolium 10 nM	13.46 $\pm$ 0.85
+ Calmidazolium 50 nM	13.11 $\pm$ 0.39
+ Calmidazolium 100 nM	13.43 $\pm$ 0.54
+ KN-62 1 μM	13.54 $\pm$ 0.40
+ KN-62 10 μM	9.43 $\pm$ 0.44
+ KN-62 50 μM	8.10 $\pm$ 0.28

* p < 0.05 vs. Control

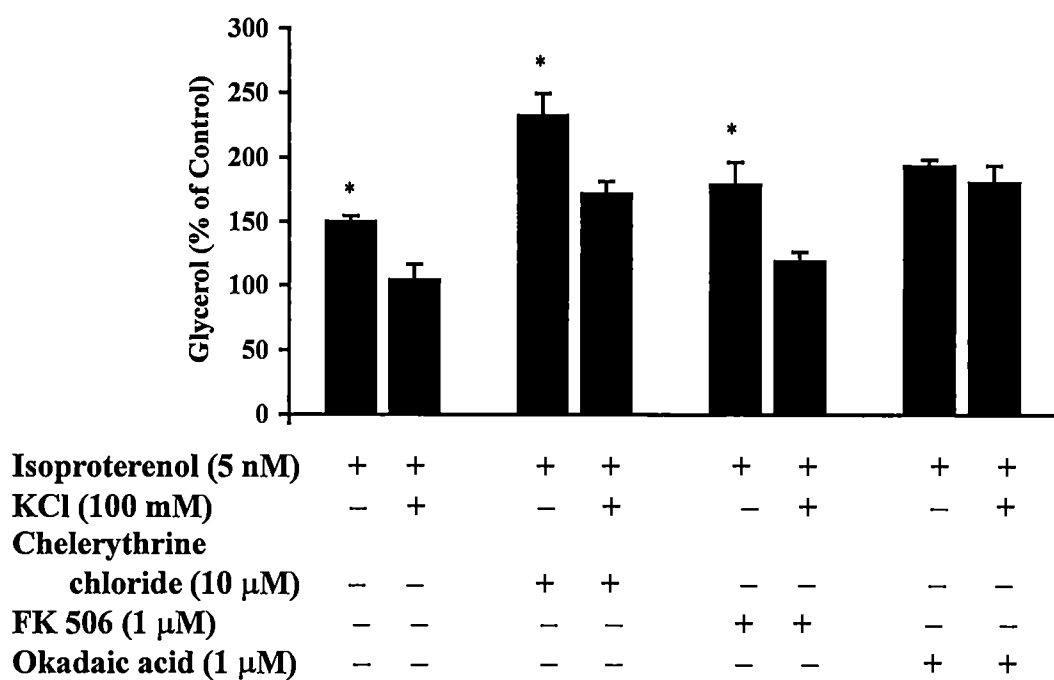


Figure 26. Effects of PKC, PP2B and PP1/2A inhibitors on the anti-lipolytic effect of KCl. Human adipocytes were treated with isoproterenol and KCl in the presence of PKC, PP2B or PP1/2A inhibitor chelerythrine chloride, FK 506 or okadaic acid for 4 hr. Data are expressed as mean $\pm$ SEM for 6 experiments. * $p < 0.05$ vs. KCl treatment.

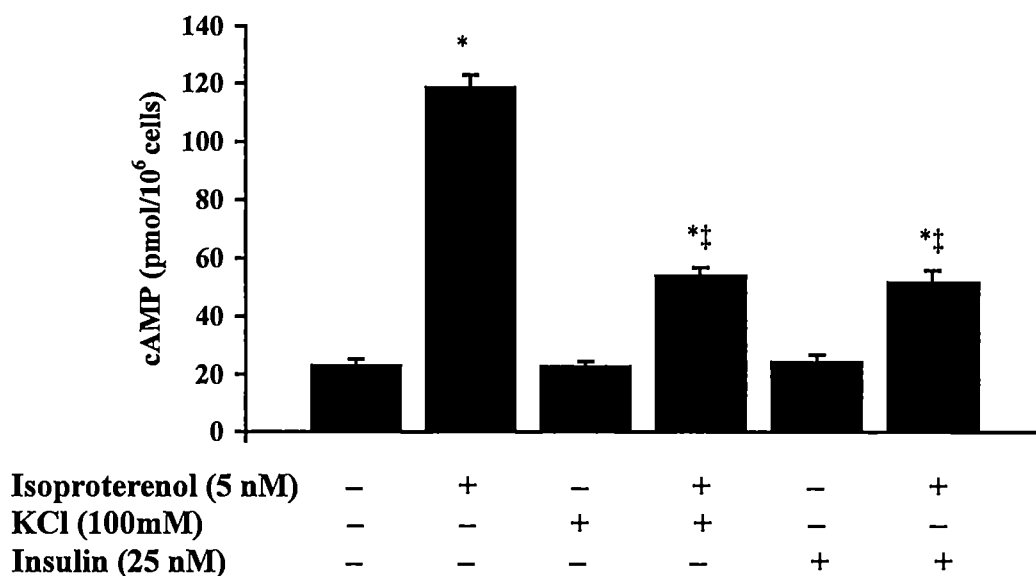


Figure 27. Effects of KCl and insulin on human adipocyte cAMP levels. Human adipocytes were treated with isoproterenol, KCl and insulin alone or isoproterenol in the presence of either KCl or insulin for 10 min. Cells were immediately lysed and cAMP content was measured using a cAMP ¹²⁵I-scintillation proximity assay (SPA) system. Data are expressed as mean $\pm$ SEM for 6 experiments. * $p < 0.05$ vs. Control. ‡ $p < 0.05$ vs. Isoproterenol treatment.

118.55 ± 4.50 pmol/ 10^5 cells). However, this was blocked by the addition of KCl and insulin to a similar extent.

HSL is regulated by reversible phosphorylation. In Figure 28A, 5 nM isoproterenol stimulated a significant increase in HSL phosphorylation in SDS-PAGE separated adipocyte phosphoproteins. However, this was prevented by the addition of either insulin or KCl. This effect was confirmed by immunoprecipitation of adipocyte phosphoproteins with anti-HSL antibody (Figure 28B).

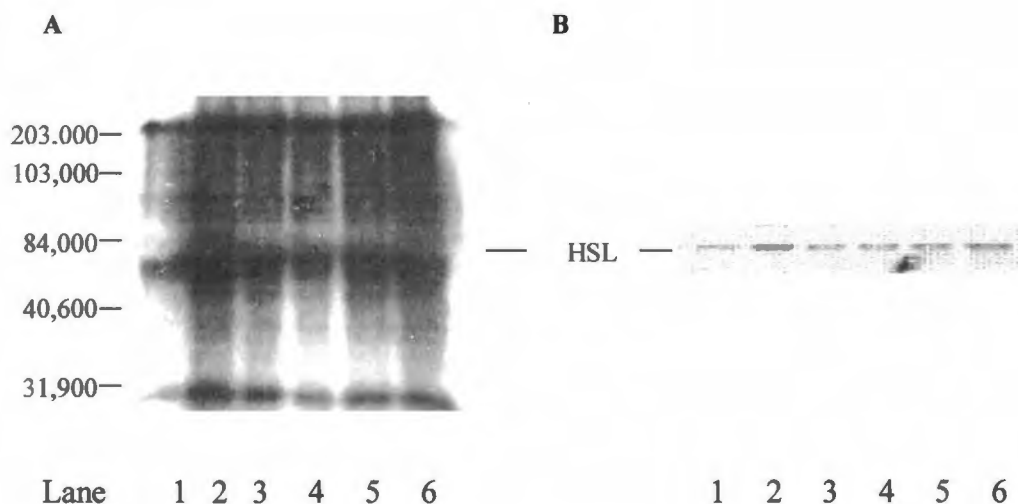


Figure 28. Effects of KCl and insulin on HSL phosphorylation. Human adipocytes were pre-labeled with ^{32}P -orthophosphate in KRH buffer with 0.3 mM KH_2PO_4 for 90 min. Cells were then treated with isoproterenol, KCl and insulin alone or isoproterenol in the presence of either KCl or insulin for 10 min. Cells were lysed in homogenization buffer. Adipocyte lysates from equal amount of cells (measured as equal amount of DNA) were either directly subjected to 10 % SDS-PAGE or incubated with anti-HSL antibody. The immunoprecipitates were washed and subjected to 10 % SDS-PAGE. Gels were stained with Coomassie Blue, dried and visualized by autoradiography. A. Adipocyte phosphoproteins separated by SDS-PAGE. B. Immunoprecipitation of adipocyte phosphoproteins with anti-HSL antibody. Lane 1: Control. Lane 2: Isoproterenol. Lane 3: KCl. Lane 4: KCl + Isoproterenol. Lane 5: Insulin. Lane 6: Insulin + Isoproterenol.

V. DISCUSSION

Several studies have reported that increasing $[Ca^{2+}]_i$ inhibits adipocyte lipolysis (1-5). However, the mechanism(s) mediating this effect remains unclear. Studies with epidermal growth factor (EGF) have shown that the anti-lipolytic effect of EGF, which is mediated through an increase in $[Ca^{2+}]_i$, primarily involves the modulation of the interaction between G_i and adenylate cyclase, leading to a reduction in cAMP (5, 6). However, studies of the lipolytic response in hypothyroid rats indicate that thyroid hormone status may modulate adipocyte lipolysis by alterations in cellular Ca^{2+} concentrations that may alter phosphodiesterase activity (4). In addition, increasing $[Ca^{2+}]_i$ by the calcium ionophore A23187 results in stimulation of phosphodiesterase and a decrease in cAMP levels (3).

In our previous study, we demonstrated that agouti protein, a murine obesity gene product, inhibited adipocyte lipolysis via a post-receptor mechanism, and that this effect is mediated by an increase in $[Ca^{2+}]_i$ (1). Further, adipose tissue from obese agouti mutants exhibits reduced lipolytic rate in response to the β -adrenergic receptor agonist epinephrine and the phosphodiesterase inhibitor theophylline, while the response to dibutyryl cAMP, a non-hydrolyzable cAMP analogue was normal (31). This suggests that increasing $[Ca^{2+}]_i$ by agouti may affect the generation or maintenance of cAMP levels, leading to inhibition of lipolysis.

In the present study, KCl was used to stimulate $[Ca^{2+}]_i$ by depolarization and was shown to exert a potent anti-lipolytic effect. KCl treatment inhibited lipolysis induced by isoproterenol, a β -adrenergic receptor agonist, forskolin, an adenylate cyclase activator, IBMX, a phosphodiesterase inhibitor and 8-bromo cAMP, a cell permeable cAMP

analogue, which activates protein kinase A. However, lipolysis induced by dibutyryl cAMP, a non-hydrolyzable cAMP analogue, was not affected by KCl, suggesting that KCl inhibition of adipocyte lipolysis may be primarily mediated by the activation of phosphodiesterase. In addition, KCl inhibition of isoproterenol-induced lipolysis was completely reversed by IBMX and dibutyryl cAMP, further confirming that the anti-lipolytic effect of KCl is primarily mediated by the activation of adipocyte phosphodiesterase and at a step before protein kinase A, as the addition of dibutyryl cAMP bypasses phosphodiesterase. Consistent with this, we demonstrated that KCl treatment significantly prevented isoproterenol stimulated cAMP production in adipocytes, leading to a reduction in adipocyte cAMP level. In addition, this results in a net decrease in HSL phosphorylation and, consequently, inhibition of lipolysis. Interestingly, this is consistent with both in vitro and in vivo data demonstrating that agouti protein inhibits adipocyte lipolysis via an increase in $[Ca^{2+}]_i$ (1). Obese agouti mutant mice express agouti protein in adipose tissue, resulting in an increase in $[Ca^{2+}]_i$ (32,33). This increase in $[Ca^{2+}]_i$ results in reduced adipose tissue lipolysis in response to β -adrenergic stimulation and phosphodiesterase inhibition, while the response to dibutyryl cAMP remains normal (31), suggesting an impairment in the maintenance of cAMP levels in these animals.

In present study, we showed that KCl inhibition of isoproterenol-stimulated lipolysis was also reversed by forskolin. This may simply be due to the high concentration of forskolin used in this study which may overwhelm the maximum capacity of adipocyte phosphodiesterase. However, a possible involvement of adenylate cyclase in the anti-lipolytic effect of KCl can not be ruled out, as the alteration in the interaction between

G_s/G_i and adenylate cyclase has been implicated in EGF's anti-lipolytic effect, which is also mediated by an increase in $[Ca^{2+}]_i$ (5,6). In addition, type V and VI adenylate cyclase have been shown to be specifically inhibited by submicromolar concentrations of Ca^{2+} (34). Further studies are needed to explore these possibilities.

Seven PDE isoforms have been discovered and relatively well-characterized (35), and recent studies have expanded this list to eleven (36). Several studies have shown that adipocytes possess several PDE isoform activity (26-28). In 3T3-L1 adipocytes and rat epididymal fat cells, a low- K_m , cGMP-inhibitable PDE isoform (Identified as PDE 3B in 37), the PDE isoform that mediates insulin's anti-lipolytic activity (30), accounts for the major particulated PDE activity (26,27). A cAMP specific PDE isoform (Identified as PDE 4 in 37) is the major cytosolic PDE isoform (26,27). In addition, the adipocyte cytosolic fraction also contains distinct Ca^{2+} /Calmodulin- (26) and cGMP-stimulated PDE activity, albeit to a less extent (26,27), that represents PDE 1 and 2, respectively, according to the nomenclature proposed in ref. 37. Human adipose tissue also contains more than one phosphodiesterase activity (28).

In the present study, we found that human adipocytes contain comparable PDE 3B and PDE 4 activity, and less PDE 1 activity, as the selective PDE 3B, 4 and 1 inhibitors cilostamide, rolipram and 8-methoxymethyl IBMX exert 2.7-, 2.2- and 40 % increase in lipolysis, respectively. However, KCl inhibition of isoproterenol-stimulated lipolysis was only reversed by the selective PDE 3B inhibitor cilostamide, suggesting that the anti-lipolytic effect of KCl is mediated by the activation of PDE 3B, the same PDE isoform mediating insulin's anti-lipolytic effect. However, we showed that the anti-lipolytic effects of KCl and insulin are additive, suggesting that KCl and insulin may activate PDE

3B via different mechanisms. Consistent with this, PI-3 kinase inhibition by wortmannin, which has been shown to be critical in insulin-induced activation of PDE 3B (38), completely abolished insulin's anti-lipolytic effect. However, it only partially blocked KCl's effect. These data further indicate a different regulatory effect of insulin and KCl on PDE 3B.

Insulin-mediated activation of PDE 3B has been shown to be dependent on PI-3 kinase (38). The generation of phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate by PI-3 kinase leads to the recruitment and phosphorylation of the serine-threonine kinase protein kinase B (PKB) by 3'-phosphoinositide-dependent kinase-1 (PDK1, reviewed in 39). PKB then phosphorylates and activates PDE 3B (9, 18, 39), leading to a reduction in cAMP and inhibition of lipolysis. However, it is not clear if KCl-mediated activation of PDE 3B also involves a phosphorylation event or another unknown mechanism. In addition, as new members of the PDE family have been identified recently (36), it is possible that KCl may activate new PDE isoforms (other than PDE 3B), which are not yet well characterized.

Calmodulin and Ca^{2+} /Calmodulin dependent protein kinases are logical targets for $[\text{Ca}^{2+}]_i$. However, to our surprise, we found that they are not involved in the anti-lipolytic effect of KCl. Nor did we find other Ca^{2+} -dependent pathways, protein kinase C (PKC) and phosphatase 2B (PP2B, calcinurin) to be involved in KCl's anti-lipolytic activity. However, the addition of okadaic acid, at a concentration that inhibits both phosphatase 1 and 2A (PP1 and 2A), completely abolished KCl's effect. This finding may suggest a possible involvement of PP1 and/or 2A in KCl's anti-lipolytic activity. Further studies

are needed to elucidate which phosphase is involved in KCl's effect and the precise role of the phosphatase in this process.

In conclusion, we demonstrated that KCl increases $[Ca^{2+}]_i$ via depolarization, which exhibits potent anti-lipolytic effects in human adipocytes. This effect is primarily mediated by activation of adipocyte phosphodiesterase and a reduction in cAMP levels, leading to a decrease in HSL phosphorylation and, consequently, inhibition of lipolysis.

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PART 7

**RELATIONSHIP BETWEEN HUMAN ADIPOSE TISSUE AGOUTI AND FATTY
ACID SYNTHASE (FAS)¹**

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I. ABSTRACT

The human homologue of the murine obesity gene, *agouti*, is expressed in adipose tissue. We have shown that recombinant agouti protein coordinately regulates adipocyte lipogenesis and lipolysis and promotes lipid storage via a Ca^{2+} -dependent mechanism *in vitro*, which may contribute to agouti-induced obesity. However, little is known about agouti's physiological function in humans. We first studied the agouti content in human mature adipocytes versus pre-adipocytes. The agouti content of human mature adipocyte was 5 times as abundant as in pre-adipocyte (19.18 ± 2.46 pg/ μg protein vs. 4.07 ± 0.51 pg/ μg protein, $p < 0.005$), suggesting that agouti is up-regulated during adipocyte differentiation. We next studied the relationship of agouti mRNA and protein to fatty acid synthase (FAS) mRNA and activity in adipose tissue obtained from non-obese and mildly obese patients (BMI range 21-31 kg/m²). Agouti protein was correlated with FAS activity ($r = 0.782$, $p < 0.005$). Similarly, human adipose tissue agouti mRNA level was also correlated to FAS mRNA level ($r = 0.846$, $p < 0.001$). These data suggest that agouti may be another adipocyte produced factor which modulates adipocyte lipid metabolism via a paracrine/autocrine mechanism.

II. INTRODUCTION

The mouse *agouti* gene encodes a 131 amino acid secreted protein that normally regulates pigment production via a paracrine mechanism during its transient expression in neonatal skin (1,2). However, ectopic expression of *agouti* in animal models such as *A^y* and *A^{vy}* or in transgenic mice overexpressing *agouti* under the control of β -actin promoter results in a yellow coat color, as well as obesity, hyperinsulinemia, insulin resistance, increased body length and susceptibility to neoplasia, which is collectively called "yellow obese" syndrome (3,4).

Much attention has been directed towards central mechanisms of *agouti*'s action. Recent evidence indicates that chronic antagonism of central melanocortin 4-receptor (MC4-R) may be responsible for the hyperphagia and obesity in yellow *agouti* mutants (5,6). However, the human homologue of *agouti* is expressed in both adipose tissue (7) and pancreas (8), where it exerts significant peripheral effects.

We have previously shown that recombinant *agouti* protein exhibits potent lipogenic and anti-lipolytic effects in human adipocytes via a calcium-dependent mechanism (9-11), and thereby promotes lipid storage in adipose tissue. Previous data from our laboratory also demonstrated that *agouti* protein stimulates calcium signaling and serves as a potent insulin secretagogue in both pancreatic β -cell line and human pancreatic islets (8). Accordingly, we propose that this *agouti*'s effect on insulin release, combined with its effect on adipocyte lipid metabolism, may represent a peripheral mechanism for *agouti* in the development of obesity. Consistent with this, although transgenic mice expressing *agouti* only in adipose tissue did not become obese, hyperinsulinemia in these animals

produced by either daily insulin injection (12) or sucrose feeding (13) did produce significant obesity in the transgenic animals, but not in their non-transgenic littermates.

However, this hypothesis is based on in vitro studies of human adipocytes and human pancreatic islets. Little is known regarding its significance in vivo.

In the present study, we investigated the relationship between agouti expression and adipocyte fatty acid synthase (FAS) expression and activity. We report here that human adipocyte agouti is up-regulated during human pre-adipocyte differentiation. In addition, there is a strong correlation between human adipose tissue agouti protein content and FAS activity as well as between human adipose tissue agouti mRNA and FAS mRNA level.

III. MATERIALS AND METHODS

A. Cell culture

Human subcutaneous pre-adipocytes were supplied by Zen-Bio (Research Triangle Park, NC). To differentiate into mature adipocytes, pre-adipocytes were plated at a density of 30,000 cells/cm² with pre-adipocyte medium [Dulbecco's Modified Eagle Medium (DMEM)/Ham's F-10 Nutrient Broth (Ham's F-10), 1:1 (v/v) containing 10 % fetal calf serum, 15 mmol/L HEPES and antibiotics] and allowed to grow confluent in 1-2 d. Cells were then induced to differentiate with differentiation medium [DMEM/Ham's F-10, 1:1 (v/v) containing 10 % fetal bovine serum, 15 mmol/L HEPES, 33 µmol/L biotin, 17 µmol/L pantothenate, 100 nmol/L insulin, 1 µmol/L dexamethasone, 0.25 mmol/L isobutyl methylxanthine, 1 µmol/L BRL49653 and antibiotics]. After three days of differentiation, cells were maintained in adipocyte medium [DMEM/Ham's F-10, 1:1 (v/v) containing 3 % fetal bovine serum, 15 mmol/L HEPES, 33 µmol/L biotin, 17 µmol/L pantothenate, 100 nmol/L insulin, 1 µmol/L dexamethasone and antibiotics] until fully differentiated.

B. Human adipose tissue

Human subcutaneous adipose tissue was obtained during liposuction from non-obese and mild obese patients, with body mass index (BMI) ranging from 21 to 31 kg/m². This protocol was approved by the Institutional Review Board for Human Subjects and the Committee for Research Participation of the University of Tennessee.

C. Agouti protein measurement

Human pre-adipocyte and mature adipocyte were rinsed once with Hank's balanced salt solution (Life Technologies, Grand Island, NT) and homogenized in sucrose buffer

[250 mmol/L sucrose, 1 mmol/L dithiothreitol (DTT), 1mmol/L ethylenediaminetetraacetic acid (EDTA) and 100 μ mol/L phenylmethylsulfonyl fluoride (PMSF)]. Human adipose tissue was homogenized in sucrose buffer. Fat-free infranatant was obtained by centrifugation at 18,500 x g for 60 min using a Sorval RMC 14 microcentrifuge (Sorval, Newtown, CT). Agouti protein was measured using whole cell homogenates from pre- and mature adipocyte preparation and the fat-free infranatant from human adipose tissue via an agouti RIA kit (Phoenix, Mountain View, CA).

D. Fatty acid synthase (FAS) activity determination

FAS activity was determined spectrophotometrically using fat-free infranatant from human adipose tissue by measuring the oxidation rate of NADPH as described previously (Moustaid et al. 1988).

E. Protein measurement

Total protein was determined by a modified Bradford method using Coomassie blue dye (Pierce, Rockford, IL).

F. Northern blot analysis

Total RNA from human adipose tissue was isolated using CsCl_2 density centrifugation. RNA from different fat samples were electrophoresed on the same gel (1 %) and transferred to nylon membranes (New England Nuclear, Boston, MA). Membranes were hybridized with ^{32}P -labeled human FAS, agouti and β -actin (Clontech, Palo Alto, CA) cDNA probe at 42°C overnight using Ultra Hyb solution (Ambion, Austin, TX). Membranes were washed with 2 X SSC at 42°C for 15-30 min, 0.1 X SSC at 42°C for 15-30 min and then 0.1 X SSC at 60°C for 30-60 min. Finally, membranes

were exposed to X-ray film (New England Nuclear, Boston, MA) at -80°C.

Autoradiograms were analyzed by densitometric scanning.

G. Statistics

All data are expressed as means $\pm$ SEM. Data were analyzed using the procedures of the SPSS Inc.(Chicago, IL). Different agouti protein content in pre- and mature adipocyte was compared by independent sample t-test. The relationship between adipose tissue agouti expression and FAS expression as well as agouti protein and FAS activity was analyzed by linear regression. Pearson correlation coefficients were calculated to determine the relationship between variables. A p value < 0.05 is considered significant.

IV. RESULTS

We first studied the effect of human adipocyte differentiation on adipocyte agouti levels. As shown in Figure 29, agouti protein level was 4-times more abundant in human mature adipocyte than in pre-adipocyte (19.18 ± 2.46 pg/ μ g protein vs. 4.07 ± 0.51 pg/ μ g protein, $p < 0.005$), suggesting that agouti is up-regulated during human adipocyte differentiation.

We next examined the relationship between human adipose tissue agouti expression and FAS expression and activity. Human adipose tissue agouti protein content varied over 10-fold among different individuals (Figure 30A). Over this range, adipose tissue agouti content was correlated with FAS activity (Figure 30A, $r = 0.782$, $p < 0.005$).

Similarly, agouti mRNA level in human adipose tissue varied over 4-fold among different individuals, and was correlated with FAS mRNA levels (Figure 30B, $r = 0.8$, $p < 0.002$).

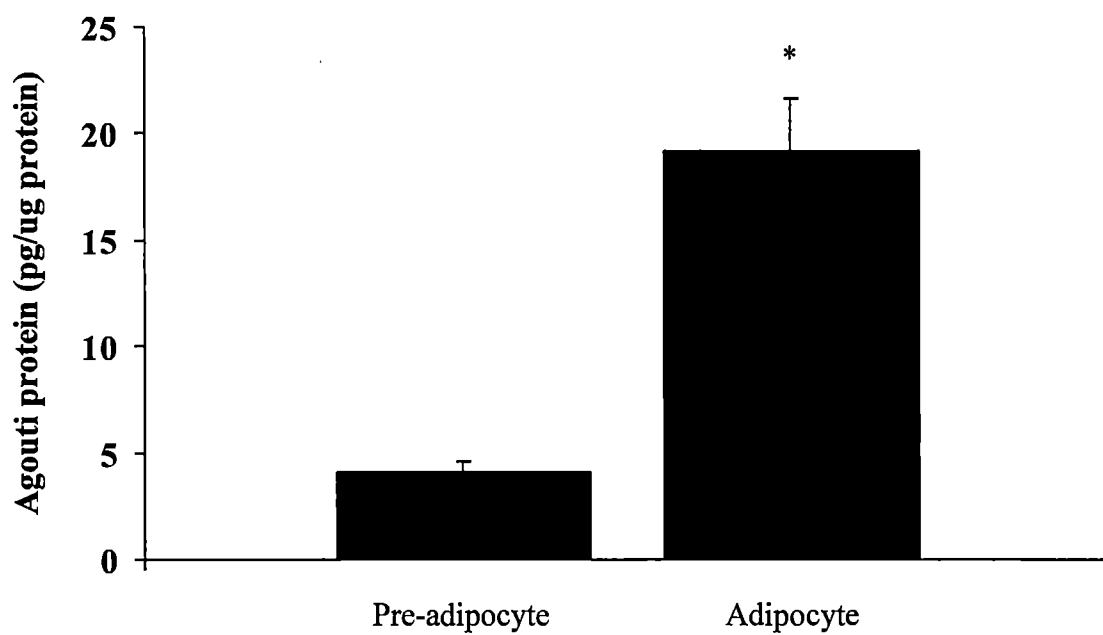
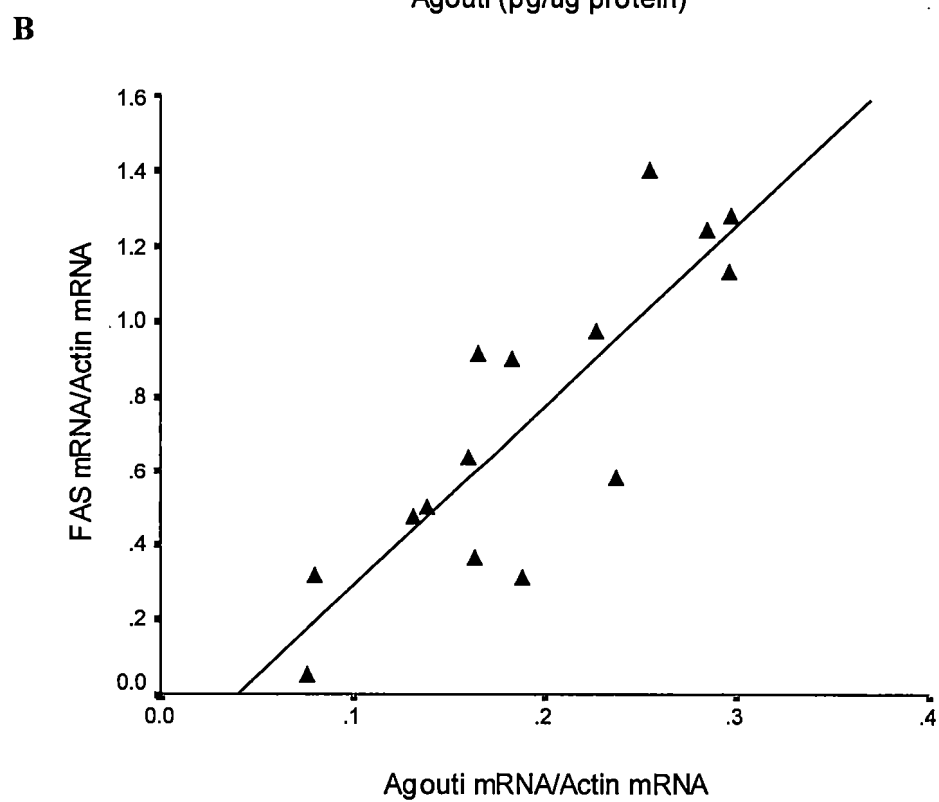
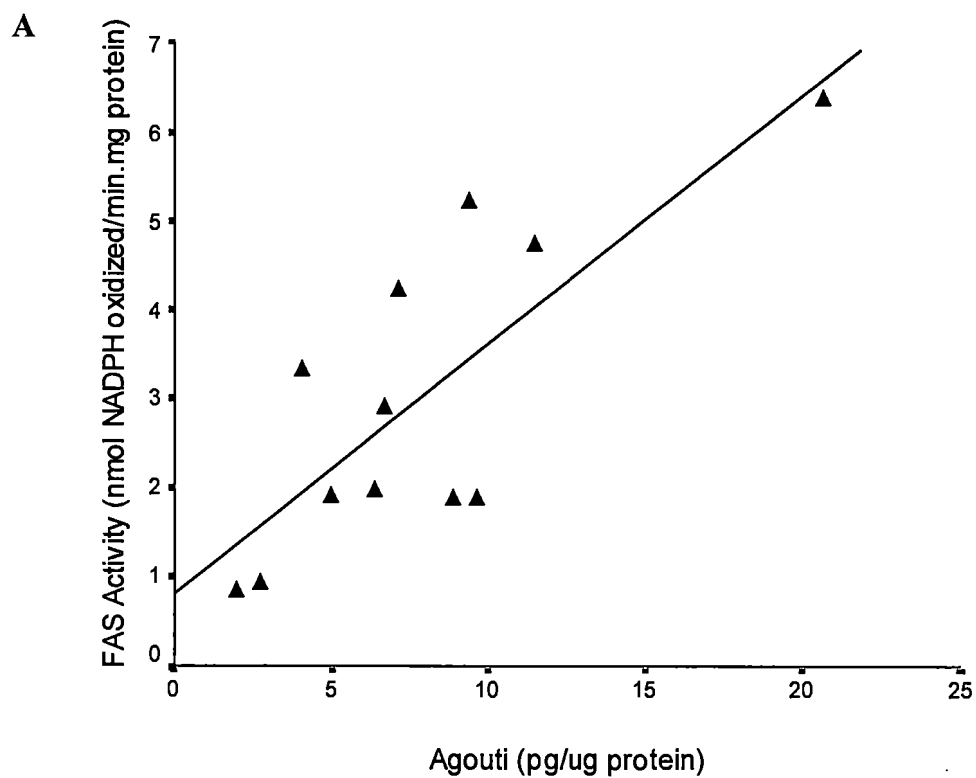


Figure 29. Effect of human adipocyte differentiation on cellular agouti protein content. *

$p < 0.005$. Values are means $\pm$ SEM, $n = 6$.

Figure 30. Correlation between agouti protein and fatty acid synthase (FAS) activity (panel A) and between agouti mRNA and fatty acid synthase (FAS) mRNA level (panel B) in human adipose tissue. Panel A: $r = 0.782$, $p < 0.005$. Panel B: $r = 0.846$, $p < 0.001$. Each data point represents a single patient. FAS: fatty acid synthase. NADPH: nicotinamide adenine dinucleotide phosphate reduced form.



V. DISCUSSION

The human homologue of *agouti*, unlike its murine counterpart, is expressed in adipose tissue (7), indicating a possible physiological role for *agouti* in human adipose tissue metabolism. We have previously shown that fatty acid synthase (FAS) mRNA level is significantly higher in adipose tissue from obese *A^y* mice than in lean (*a/a*) controls (9). In addition, recombinant *agouti* protein stimulated both the expression and activity of adipocyte FAS *in vitro* via a Ca^{2+} -dependent mechanism (9). Further, the FAS promoter contains an *agouti* response element (9) that also responds to KCl, a membrane depolarization agent (unpublished observations). We have also demonstrated that *agouti* protein inhibited both basal- and agonist-stimulated lipolysis in human adipose tissue via a Ca^{2+} -dependent mechanism (11). These *in vitro* data suggest a coordinated regulation of adipocyte lipid metabolism by *agouti*, which may in turn promote lipid storage in adipose tissue.

Numerous reports have demonstrated that adipose tissue synthesizes various proteins and hormones, participating in energy regulation through a network of endocrine, paracrine and autocrine signals (16,17). Leptin, for example, is a satiety factor produced by adipocyte and regulates body weight through its effects on food intake in hypothalamus. In addition, adipose tissue has been also shown to produce factors that act in endocrine and paracrine/autocrine manner to modulate its own metabolism, pre-adipocyte proliferation and differentiation, and multiple systemic effects. These factors include cytokines, interleukin-6 and tumor necrosis factor- α (TNF- α), acylation-stimulating protein (ASP), angiotensin II, prostaglandins, sex steroids, plasminogen activator inhibitor-1 (PAI-1), etc (16-18).

Fatty acid synthase (FAS) is the key enzyme in *de novo* lipogenesis. Both human liver and adipose tissue exhibits substantially lower FAS activity than found in rats (19). None the less, significant *de novo* lipogenesis is well documented in human adipocytes. Human adipocytes contain substantial levels of FAS expression and activity that is sensitive to both nutritional and hormonal modulation (14, 20, 21). Studies in humans given a high carbohydrate diet demonstrated that total body fat synthesis significantly exceeded hepatic *de novo* lipogenesis, implicating that adipose tissue may be the major site for fat synthesis (22). Further, another study showed that adipose tissue accounts for up to 40% of whole body lipogenesis under this condition (23). The effect of carbohydrate intake on adipose tissue lipogenesis is qualitatively similar in humans and in rats. Quantitatively, rats have higher rates of lipogenesis, which may be explained, in part, by the higher metabolic rate in rat than in human (23), or by higher fat content in some human diets, as high fat diets suppress lipogenesis. Moreover, FAS levels are elevated in obese animals (24). Thus, induction of adipocyte lipogenesis may contribute to obesity.

In the present study, we demonstrated that human adipocyte agouti is up-regulated during differentiation. In addition, we have shown a strong correlation between agouti expression and FAS expression and activity in human adipose tissue. It is possible that agouti and FAS are regulated in a similar manner. FAS is primarily regulated by hormonal and nutritional factors at the transcription level. It is increased by feeding, thyroid hormone and insulin, and decreased by starvation, cAMP and polyunsaturated fatty acids (25). However, it is currently unknown which factor(s) may modulate adipocyte agouti expression and how it is regulated. Further, as agouti is a

paracrine/autocrine factor which does not enter general circulation (26), and as we have shown agouti to regulate FAS expression and activity *in vitro* (9), it is possible that agouti may be another adipocyte synthesized factor, which may contribute in part to the regulation of adipocyte lipid metabolism.

In summary, we have found that human adipocyte agouti is up-regulated during adipocyte differentiation. In addition, there is a strong correlation between human adipose tissue agouti protein content and FAS activity as well as between human adipose tissue agouti mRNA and FAS mRNA level. Consistent with this, we have shown agouti to regulate adipocyte lipid metabolism *in vitro* (9,11). These data suggest that agouti may be an additional adipocyte factor, which modulates adipocyte lipid metabolism via a paracrine/autocrine mechanism.

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PART 8
SUMMARY AND CONCLUSION

SUMMARY AND CONCLUSIONS

In summary, these studies demonstrated that agouti modulates both pancreatic islet and adipocyte function via a Ca^{2+} -dependent mechanism (Figure 31).

We have demonstrated that the human homolog of agouti is expressed in human pancreas, and increases $[\text{Ca}^{2+}]_i$ in human pancreatic β -cells via stimulating Ca^{2+} influx. In addition, agouti protein potently stimulates both insulin and amylin release from human pancreatic islets via a Ca^{2+} -dependent mechanism. These findings suggest that agouti may act in a paracrine/autocrine manner to modulate β -cell function. Agouti stimulation of insulin and amylin release may serve as a compensatory system in regulating glucose homeostasis. However, agouti stimulation of insulin release may contribute to the hyperinsulinemia commonly present in yellow obese mice and obese humans, and contribute to insulin resistance and obesity. In addition, the ability of agouti to stimulate amylin release may predispose individuals to the development of islet amyloidogenesis, leading to reduced β -cell mass, impaired β -cell function and diabetes.

In adipocytes, we have previously demonstrated that agouti upregulates the expression and activity of fatty acid synthase (FAS), a key enzyme in *de novo* lipogenesis, via a Ca^{2+} -dependent mechanism. Agouti also potently inhibited basal and agonists stimulated lipolysis in human adipocytes in a Ca^{2+} -dependent manner. This represents a coordinated regulation of agouti on adipocyte lipid metabolism, thereby promoting lipid storage in adipose tissue. The mechanism of inhibition of adipocyte lipolysis by agouti mediated increase in $[\text{Ca}^{2+}]_i$ appears to involve the activation of adipocyte phosphodiesterase, resulting in a decrease in cAMP levels and a net decrease in the phosphorylation of hormone sensitive lipase, a key enzyme in lipolysis.

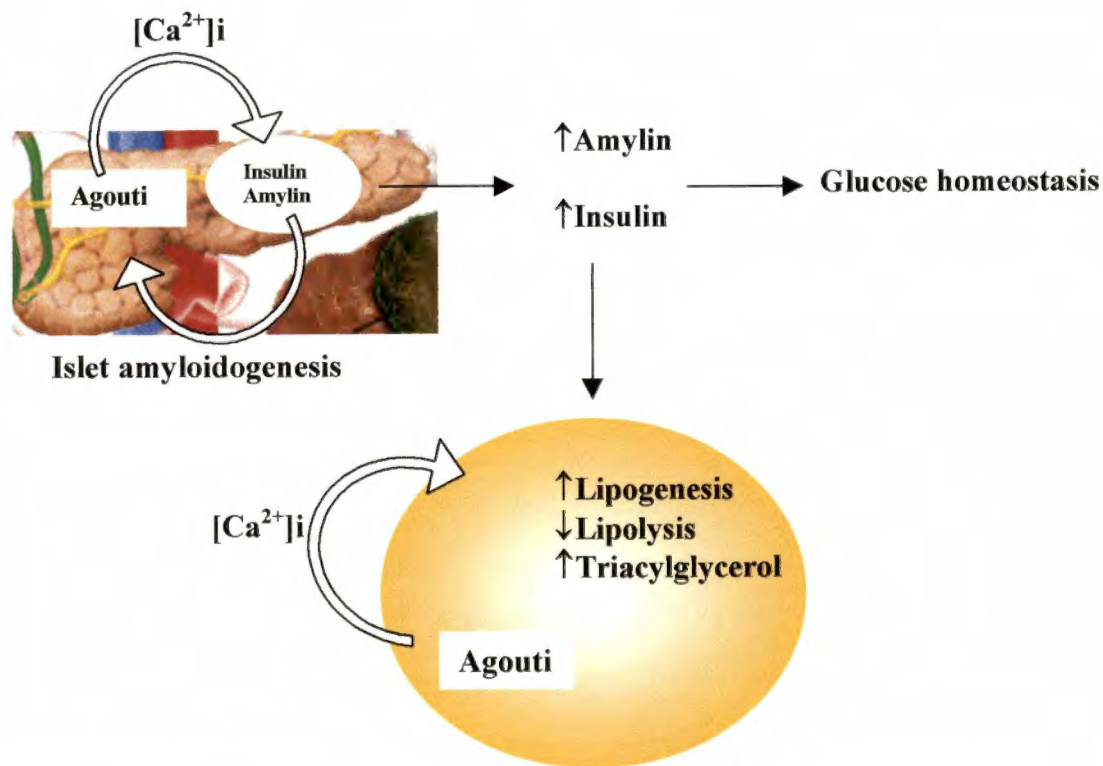


Figure 31. Role of agouti in obesity: modulation of pancreatic β -cell and adipocyte function via a Ca^{2+} -dependent mechanism. Agouti is expressed in both human pancreas and adipocytes and acts in a paracrine/autocrine manner to stimulate insulin and amylin release in pancreatic β -cell and coordinately regulates lipid metabolism in adipocytes via a Ca^{2+} -dependent mechanism. Agouti stimulation of insulin and amylin release may serve as a compensatory mechanism in regulating glucose homeostasis. However, agouti stimulation of insulin release may contribute to hyperinsulinemia and may act synergistically with agouti in adipocytes to modulate lipid metabolism, thereby promoting obesity. In addition, agouti stimulation of amylin release may predispose individuals to islet amyloidogenesis, leading to impaired β -cell function and diabetes.

In addition, our data suggest that agouti stimulated insulin release may act synergistically with agouti in adipocytes to modulate adipocyte lipid metabolism, thereby contributing to obesity.

Consistent with our in vitro data of agouti regulating adipocyte FAS, we have found a positive correlation between adipose tissue agouti protein level and FAS activity as well as between adipose tissue agouti mRNA and FAS mRNA levels. It is possible that agouti, which is produced by adipocytes, may act in a paracrine/autocrine manner to modulate adipocyte function, and partly contribute to the regulation of adipocyte fatty acid synthase.

In conclusion, our present studies has defined a peripheral model of agouti action in the development of obesity. However, unlike most rodent obese models which are caused by single gene mutations, human obesity is a multifactorial disorder, which is influenced by both genetic and environmental factors and involves both gene-gene and gene-environment interactions. It is more challenging to define the causative mechanisms in human obesity. Nonetheless, studying the genetics and physiology of rodent single gene obesity models will provide new insight into the mechanisms of the development of obesity and search for therapeutic targets in treating obesity.

VITA

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