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Effects of Agouti Related Protein and Androgens on Growth

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ABSTRACT

A dominant mutation at the agouti locus on mouse chromosome 2 is a promoter of overeating and increased linear growth. Agouti related protein, which can be produced using bacteria can be used to reproduce the same growing effect. Androgens are known to promote leanness and increased growth. Combined effects of testosterone and agouti may provide fast growing-lean animals.

Key words: Agouti, mice, ARP, testosterone, growth, leanness

INTRODUCTION

The agouti gene is related to coat color regulation in mice, with temporary expression in the newborn mice, resulting in a pigmentation pattern classically called agouti. Agouti coats are classified by a predominantly yellow fur. In wild-type mice, the agouti gene is expressed only for a short period, during neonatal growth, resulting in the agouti pigmentation pattern (Yen *et al.*, 1994). Therefore, it should be appropriate to use the agouti gene in newborn, even further to increase weight gain. Agouti related protein has been synthesized (Rosenfeld *et al.*, 1998) and it can be used along with androgens to promote weight gain.

Agouti gene encodes a protein containing 131 amino acids. Normally, it is expressed in hair follicles, where it induces melanocytes within hair follicles to synthesize the yellow pigment that characterizes the predominantly yellow fur. Agouti protein is also expressed in testes and skin during embryonic development. Dominant mutations at the agouti gene cause expression of agouti protein in every tissue, not just in skin and testes (Roberts and Greenberg, 1996). The mutation occurs in an allele at the agouti locus on mouse chromosome 2 (Bultma *et al.*, 1992). Analysis of dominant agouti alleles A^{y}, A^{vy}, A^{iapy}, A^{sy}, A^{iy} and A^{hvy} showed that mutations in the promoter region of agouti gene causes excess expression of normal agouti transcripts in all tissues throughout life (Zernel and Bingzhong, 1998). All six of the autosomal dominant yellow mutations in the agouti (A) gene (e.g. A^{y}, lethal yellow; A^{vy}, viable yellow; A^{sy}, sienna yellow; A^{iy}, intermediate yellow; A^{hvy}, hypervariable yellow; A^{iapy}, intracisternal A particle yellow) are promoter mutations characterized by obesity, hyperphagia, hyperinsulinemia, hypercorticosteronism and increased linear growth (Yen *et al.*, 1994). The degree of obesity correlates with the amount and intensity of the yellow hair pigment, which results from over expression of the agouti gene (Leibel *et al.*, 1997). Hyperphagia is a major contributor to the obesity, since food restriction to 80% of normal intake can decrease body weight and body fat content to near ad libitum levels (Yen *et al.*, 1994). It is logical to suggest that metabolic alterations increase metabolic efficiency and/or partitioning to adipose tissue of the yellow mutants. Because restriction to 60% of normal intake does not decrease fractional body fat content beyond the reduction induced by the restriction to 80% of usual calories. Unlike the Lep^{ob} or $Lepr^{db}$ mice, the yellow mutants display no defect in temperature control under cold stress (Leibel *et al.*, 1997).

Melanocyte Stimulating Hormone (MSH, Melanocortin)

Melanocortin in General

The term melanocortin refers to several of the post-translational products of the proopiomelanocortin gene, adrenocorticotropic hormone (ACTH) (De Wied *et al.*, 1982) and α-, β- and γ-MSH. Besides the well known effects of these peptides on adrenal cortical steroidogenesis (ACTH) and pigmentation (α-MSH and ACTH), melanocortins have also been involved in learning and memory, blood pressure control, immune modulation and weight homeostasis among others (De Wied *et al.*, 1982 and Fan *et al.*, 1997b).

Agouti peptide, normally only found in the skin, is a high-affinity antagonist of the melanocyte-stimulating hormone receptor (MC1-R), causing an inhibitory effect on eumelanin pigment synthesis. The agouti peptide is also an antagonist of the hypothalamic melanocortin-4 receptor (MC4-R) (Fan *et al*., 1997b).

MC-4 and Other MSH Related Receptors

The melanocortin-4 receptor (MC4-R) is a G protein-coupled, seven-transmembrane receptor expressed in the brain. Inactivation of this receptor by gene targeting results in mice that develop adult onset obesity associated with hyperphagia, hyperinsulinemia and hyperglycemia. This syndrome repeats several characteristic features of the agouti obesity syndrome concisely, which is due to excessive expression of agouti protein. Data identify a novel signaling pathway in the mouse for body weight regulation and suggest that the primary mechanism by which agouti induces obesity is chronic antagonism of the MC4-R (Huszar *et al*., 1997).

Mouse agouti protein is a paracrine-signaling molecule that has previously been shown to be an antagonist of melanocortin action at several cloned rodent and human melanocortin receptors (Yang *et al*., 1997). Agouti-signaling protein (ASIP), the human homolog of mouse agouti may be stably expressed in L cells (hMC1R, hMC3R, hMC4R, hMC5R) or in the adrenocortical cell line OS3 (hMC1R, hMC2R, hMC4R). Purified recombinant ASIP inhibits the production of cyclic AMP stimulated by alpha-MSH (hMC1R, hMC3R, hMC4R, hMC5R) or by ACTH (hMC2R). However, the degree of ASIP inhibition varies significantly among the receptor subtypes; ASIP is a potent inhibitor of the hMC1R, hMC2R and hMC4R, but has relatively weak effects at the hMC3R and hMC5R. The apparent mechanism of ASIP antagonism varies among receptor subtypes also, with characteristics consistent with competitive antagonism being observed only at the hMC1R and more complex behavior observed at the other receptors. ASIP inhibition at these latter receptors, nonetheless, can be classified as surmountable (hMC3R, hMC4R and hMC5R) or nonsurmountable (hMC2R). Recombinant ASIP also inhibits binding of radiolabeled melanocortins, [125I-Nle4, D-Phe7] alpha-MSH and [125I-Phe2, Nle4] ACTH 1-24, to the hMCR 1-5 receptors, with a relative efficacy that paralleled the ability of ASIP to inhibit cAMP accumulation at the hMC1R, hMC2R, hMC3R and hMC4R (Yang *et al*., 1997).

Agouti Related Protein (ARP)

ARP in General

Agouti protein and Agouti-related protein (ARP) are paracrine-signaling molecules that normally regulate pigmentation and body weight, respectively (Ollman *et al*., 1998). The agouti-related protein gene is a newly discovered gene involved in the control of feed intake. Excess expression of ARP results in obesity and diabetes. Data suggest a role for the ARP in the regulation of MSH receptors expressed in the central nervous system. The availability of recombinant protein is required to fully address this potential interaction. Rosenfeld *et al*. (1998) reported that a nearly full-length form of ARP (MKd5-ARP) was expressed in the *Escherichia coli* and appeared as large intermolecular disulfide-bonded aggregates. Following oxidation, refolding and purification, this protein was soluble. The purification to homogeneity of a second, truncated form of ARP (Md65-ARP) which was expressed in the insoluble or inclusion body fraction was also achieved (Rosenfeld *et al*., 1998). Recombinant Agouti-related protein is a potent, selective antagonist of Mc3r and Mc4r, MSH receptor subtypes involved in weight regulation (Ollman *et al*., 1997). The demonstration that ARP is an endogenous antagonist with respect to these receptors is a unique mechanism within the central nervous system and has important implications in the control of feeding (Rosenfeld *et al*., 1998).

Agouti-related protein, also known as agouti-related transcript (16q22 in human; 8D1-D2 in mouse), is a 132-aa protein 25% identical to agouti. It is expressed mainly in the adrenal gland and the arcuate nucleus of the hypothalamus. The gene is overexpressed in Lep^{ob} and $Lepr^{db}$ homozygotes and ARP selectively antagonizes α-MSH binding to MC3R and MC4R. Thus, ARP acting via MCRs is a potential distal mediator of leptin effects on energy homeostasis (Ollmann, 1997 and Shutter *et al*., 1997).

The Mechanism Through Which ARP Works

The exact mechanism by which Agouti protein and ARP inhibit melanocortin-receptor signaling is not completely clear. *In vitro,* recombinant Agouti protein will inhibit binding of radiolabeled melanocortins to cells with melanocortin receptors (Lu *et al*., 1994 and Yang *et al*., 1997). Ollman *et al*. (1998) suggested that Agouti protein binds MSH receptors and acts as a competitive antagonist of ligand binding, although Agouti protein and melanocortin peptides exhibit no sequence similarity. Studies of Agouti protein in B16 melanoma cells supported this logic. Dose-response curves of α-MSH stimulated cyclic-AMP accumulation in the presence of Agouti protein were consistent with simple competitive antagonism (Blanchard *et al*., 1995 and Williard *et al*., 1995). Additional

reports speculate that Agouti protein promotes MSH receptor internalization, or it functions as an inverse agonist of melanocortin receptors (Siegrist *et al.*, 1996 and 1997). B16-G4F cells, a cell variant that lacks the MC1 receptor, did not respond to agouti (Siegrist *et al.*, 1997).

To test the hypothesis that agouti causes obesity by antagonism of hypothalamic melanocortin receptors, scientists identified cyclic melanocortin analogues that are potent agonists or antagonists of the neural MC3 and MC4 receptors. Intracerebroventricular administration of the agonist, MTII, inhibited feeding in four models of hyperphagia: fasted C57BL/6J, ob/ob and A^Y mice and mice injected with neuropeptide-Y. Co-administration of the specific melanocortin antagonist and agouti-mimetic SHU9119 completely blocked this inhibition. Moreover, administration of SHU9119 significantly enhanced nocturnal feeding, or feeding stimulated by a prior fast. The data show that melanocortinergic neurons inhibit feeding behavior. Chronic disruption of this inhibitory signal causes the agouti obesity syndrome (Fan *et al.*, 1997b). From all of these results, it can be concluded that agouti is an antagonist, or inverse agonist, acting through the MC receptors.

In addition of binding to MSH receptors, it is discovered that dominant agouti mutants display increased $[Ca^{2+}]I$ in most tissues, causing increased vascular reactivity and insulin resistance in vascular smooth muscle and skeletal muscle cells, respectively. Further, it was discovered that recombinant agouti protein directly increased $[Ca^{2+}]I$ in a variety of cells and stimulated both the expression and activity of adipocyte fatty acid synthase and increased triglyceride accumulation in a (Ca^{2+})-dependent manner. These effects can be mimicked by stimulation of Ca^{2+} influx and blocked by Ca^{2+} channel inhibition, while treatment of mice with a Ca^{2+} antagonist decreases agouti-induced obesity (Zemel, 1998).

The lethal yellow (A^Y/a) mouse is resistant to the leptin weight-reducing effects. It has been thought that those effects of leptin are transmitted essentially by way of proopiomelanocortin (POMC) neurons. However, the central effects of defective POMC signaling and the absence of leptin, on weight gain in double-mutant lethal yellow (A^Y/a) leptin-deficient (lep^{ob}/lep^{ob}) mice were shown to be independent and additive. Moreover, deletion of the leptin gene restores leptin sensitivity to the agouti mice. This suggests that in the A^Y/a mouse, obesity is independent of leptin action and resistance to leptin is due to desensitization of leptin signaling (Boston *et al.*, 1997).

Agouti protein in the hair follicle changes pigment synthesis from eumelanin (black) to pheomelanin (yellow) production by blocking the action of MSH at its receptor (MC1R). Obesity induction by the yellow mutations does not require the MC1R receptor since Ay e/e mice (lacking extension (e) = Mc1r) are still obese (and black) (Poole and Silvers, 1976). Agouti protein synthesized in the brains of mutant animals must be affecting the animal using another pathway since the Mc1r gene product is not required for A^y-induced obesity. Recently, evidence is found that melanocortin 4 receptor (MC4R) in the brain mediates food intake. Knockout animals homozygous for a disruption of the Mc4r gene are as obese as A^y animals (Fan *et al.*, 1997a and Huszar *et al.*, 1997). The normal ligand(s) for MC4R is not known. However, ARP competes with high affinity against MSH at MC4R (Yen *et al.*, 1994). Thus, it appears likely that some of the obesity-producing effects of ARP expression in the brain may be due to its interference with signal generation by MSH at MC4R, a signal which normally acts to suppress food intake. ARP also appears to induce lipogenesis in adipocytes by enhancing insulin sensitivity via a pathway that increases intracellular calcium (Manne *et al.*, 1995).

Neuropeptide-Y (NPY) and ARP Comparison in Terms of Increasing Feed Intake

In spite of evidence that Neuropeptide-Y plays a big role in stimulating appetite, NPY-deficient mice eat normally, grow normally and refeed after a fast normally. In addition, all endocrine responses to fasting are normal (Palmiter *et al.*, 1998). Agouti mice keep their fat content in a certain level even when food is restricted to 60 % of normal (Yen *et al.*, 1994). The response of NPY-null mice to diet-induced obesity, chemically induced obesity (monosodium glutamate and gold thioglucose) and genetic-based obesity (lethal yellow agouti, A^y) are all normal. However, NPY deficiency does partially reduce the obesity and all of the adverse endocrine effects of leptin deficiency in ob/ob mice. NPY-null mice as well as mice deficient in both NPY and leptin are more sensitive to leptin, suggesting that NPY may normally have a restorative inhibitory action on signals by leptin that give the full feeling. NPY-null mice display the normal increased feeding response to injected NPY. Thus, the only condition where a role for NPY in body-weight regulation is observed is in the context of complete leptin deficiency, where absence of NPY is beneficial (Palmiter *et al.*, 1998).

Leptin or ARP?

Genetic obesity is associated with increased neuropeptide-Y (NPY) messenger RNA (mRNA) and decreased POMC mRNA in the hypothalamus of ob/ob and db/db mice, or impaired sensitivity to alphaMSH (derived from POMC) in the yellow agouti mouse (Bergen *et al.*, 1998). One could suggest using leptin instead of ARP since leptin

knockout mice are actually larger than agouti mice. There is a straight logic in that suggestion; however, the use of testosterone will change the matter. It is very well known that testosterone increase aggressiveness in most of the animals, including mice and ruminants. Large doses of testosterone, therefore, can be dangerous to animals themselves they will fight more and to the humans who take care of those animals, especially in cattle. The use of testosterone is essential since it will increase the lean tissue, decrease adipose tissue, providing the "lean growth" humans are looking for. The extra food animals eat will go to muscle production, instead of being wasted in production of fat, which is cheap and light compared to muscle tissue.

Leptin mutations result in hyperhagia, hyperinsulinemia and early-onset obesity that are hard to reverse. However, agouti mutant mice will advance to be obese slowly, similar to adult-onset obesity in humans (Leibel *et al*., 1997). Leptin mutants have a defect in thermogenesis detectable within the first few days of life. They have a lower core temperature and a more rapid decline in body temperature when exposed to cold stress (Himms-Hagen, 1985).

Testosterone Effects on Growth

Testosterone effects have been known for a long time. In a report by Rooyackers and Sreekumaran, (1997), it is stated that anabolic effect of testosterone on skeletal muscle involving hypertrophy is evident (Mooradian *et al*., 1987). Increased protein synthesis is the main cause of the anabolic effect (Mooradian *et al*., 1987 and Florini, 1970); while insulin decreases protein breakdown (Rooyackers and Sreekumaran, 1997). Since animals producing excessive amounts of agouti protein have hyperinsulinemia, they should keep some amount of muscle tissue in addition to the fat. One could suggest that insulin desensitization in receptors would prevent the effects of insulin to increase muscle tissue production. This bears some truth. However, animals do not become diabetic immediately and until they do, they will benefit from high insulin. In addition, a recent report stated that ARP appears to enhance insulin sensitivity (Manne *et al*., 1995). Giving testosterone should also increase the muscle tissue in massive amounts, since most of the food taken will have a chance to be converted to skeleton muscle.

In a study, neonatal castrated pigs weighed 85 and 76% of that of intact males at 7 and 21 days, respectively, which illustrates that castration will decrease growth dramatically (Mulvaney *et al*., 1988). It has also been known that injection of testosterone to pigs at birth increases weaning weight and survival during the neonatal period (Dvorak, 1981).

Testosterone is expected to increase aggressiveness. However, recent research shows that ARP is competitive with ACTH. This may decrease, even counteract the dangers of nervousness testosterone causes. Zemel (1998) reported that short-term agouti treatment totally inhibited ACTH-induced lipolysis ($P < 0.05$).

Combined Effects of Testosterone and Agouti

ARP increases feed intake, thus increasing linear growth (Yen *et al*., 1994). Testosterone increases lean tissue as well as accelerating linear growth (Arnold, 1997). This should provide the animals with a great amount of linear growth. Testosterone is expected to provide the necessary signal to convert the food to muscle tissue, rather than convert it to fat. Since muscle is heavier than fat and spans more space, weight and size of the animals are expected to be bigger than normally they would have been if they were only given ARP. However, using testosterone may inhibit LH and thus, delay puberty in females. Blocking puberty and pregnancy should be a benefit to feedlot producers.

T_3 and T_4 increases when animals eat excessively, as in the agouti case. The thyroid hormones increase the number of adrenaline receptors (Beta adrenergic receptors) and thus, adrenaline (epinephrine) response increases to a stress situation. If there are steroids in the environment, epinephrine increases GH, thus IGF. If the animals are given testosterone, GH levels should increase in any stress situation. Thyroid response to GH increases if there are steroids in the environment. Thus, metabolic rate and leanness increases.

Using testosterone on subordinate animals will increase their social status. It is known as a common knowledge that bigger animals and animals with higher androgens have higher social status and thus, they are able to eat more. This causes the bigger animals to be bigger. One of the most important effects for the animals to be in a higher status in the animal hierarchy is the size. One could easily suggest that size be mostly determined by the steroid production, since steroids elevate GH, thus the IGF levels. It could be suggested that increasing androgen levels in all of the animals would boost the behavior that causes increase in social status in all of the animals, thus, having no effect on the ranking. However, it is quite possible that extra dose of testosterone will fill up all the receptors, especially in young animals, taking all animals to an equal space in the hierarchy, providing a lot more standardization. It is known that standard products will bring ease to processing, causing the processing plants to pay more to the producers with more standard products.

Animals given ARP tend to eat more and use most of the energy on growth, rather than metabolism and daily activities. Animals given androgens tend to have leaner tissue, increased growth hormones and a higher level of metabolism. With the increased food intake due to ARP and leanness due to androgens, producers can have heavier and leaner animals.

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