Caloric Restriction Abolishes Enhanced Metabolic Efficiency Induced by Ectopic Agouti Protein in Yellow Mice (44390)

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Abstract. **Caloric restriction (CR), from approximately 3 months of age, at 70% of the *ad libitum* (AL) caloric intake prevented development of overt obesity in female "viable yellow" A^{vy}/A (BALB/cStCrlfC3Hf/Nctr × VY/WffC3Hf/Nctr-A^{vy}) F_1 hybrid mice. In adult yellow A^{vy}/A mice, caloric restriction eliminated the increased metabolic efficiency associated with the presence of agouti protein in ectopic sites. At 4 weeks of age, the yellow A^{vy}/A mice were ≈ 14% heavier and by 12 weeks of age, when caloric restriction began, they were ≈ 24% heavier than the congenic agouti *A/a* mice. Between 4 and 12 weeks, the yellow mice gained ≈ 63% in body weight, whereas the agouti mice gained only ≈ 44%. While the comparable AL A^{vy}/A mice gained ≈ 128% and the AL *A/a* mice gained ≈ 41% between 12 and 51 weeks of age, the CR A^{vy}/A and *A/a* mice gained only 16% and 15%, respectively. Mean brain weights of CR mice of both genotypes were lower than those of the comparable *ad libitum*-fed (AL) groups; however, CR A^{vy}/A mice had slightly, but significantly ($P < 0.0001$), higher brain weights than CR *A/a* mice. The larger mean brain weight and retention, during caloric restriction, of the somewhat greater prerestriction A^{vy}/A mean body weight compared with prerestriction *A/a* mice were consonant with the hypothesis that ectopic agouti protein affects somatic growth directly or indirectly. This may be related to altered developmental/metabolic programming in yellow mice, indicated by greater metabolic efficiency and by an early transient increase in circulating IGF-1 levels. The specific cellular processes modulated by the agouti protein in ectopic sites remain to be identified.**

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Caloric restriction of "viable yellow" (A^{vy}/-) mice from ≈ 3 months of age prevents or reverses overt obesity, but does not reverse the already expressed tendency to a larger body mass. As part of a study to characterize transcriptional regulation of diet-regulated genes, we determined body and brain weights of yellow A^{vy}/A and agouti *A/a* (BALB/cStCrlf C3Hf/Nctr × VY/WffC3Hf/Nctr-A^{vy}) F_1 female mice that had been fed *ad libitum* (AL) or had been restricted (CR) to 70% of the AL caloric intake.

Viable yellow mice start to become obese, hyperinsulinemic, and mildly hyperglycemic around puberty, whereas their agouti siblings remain lean, normoinsulinemic, and normoglycemic. Since the F_1 hybrid background genome of these two genotypes was identical, except for the A^{vy} allele, any differences in the response of any diet-regulated genes could be ascribed to the physiologic/metabolic effects of the A^{vy} mutant gene.

The A^{vy} mutation arose through spontaneous insertion of a single intracisternal A particle (IAP) sequence in the *agouti* gene preceding the first coding exon (1). The IAP promoter/enhancer sequence is constitutively active in essentially all cells and tissues. In yellow A^{vy}/A mice transcription of the gene is under control of the IAP promoter/

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enhancer so that it is transcribed continuously in all tissues. In contrast to agouti *A/a* mice in which the agouti protein is transcribed only in skin during the short period while the subapical yellow band in the hair is formed, it is expressed ubiquitously and continuously in A^{vy}/A tissues (1). This continuous ectopic expression results not only in the yellow hair, but also in obesity, hyperinsulinemia, diabetes, increased somatic growth, and increased susceptibility to hyperplasia and tumorigenesis (2, 3).

The agouti protein binds with high affinity to the α-melanocyte stimulating hormone (α-MSH) receptor MC1-R (melanocortin-1 receptor) and to another melanocortin receptor, MC4-R, located in the brain (4, 5). By inhibiting binding of α-MSH to its receptors, the agouti protein prevents activation of adenylyl cyclase and the subsequent elevation of c-AMP concentration in melanocytes and other cells.

Ectopic expression of the agouti protein also has been reported to elevate intracellular Ca^{2+} concentrations in soleus and gastrocnemius muscles of mottled yellow A^{vy}/a mice. This action of agouti protein was confirmed by the observation that it increased Ca^{2+} influx in L6-cultured myocytes and in soleus myocytes from *a/a* mice (6).

Although there are regulatory interactions between adenylyl cyclase activation and intracellular cyclic AMP and Ca^{2+} concentrations (7), a specific functional relation between the effects of the agouti protein on these two parameters is unknown; however, it seems reasonable, as a minimalist working hypothesis, that the mode(s) of action of the agouti protein is(are) similar in most cell types.

The presence of the agouti protein in ectopic sites results in adult onset obesity and diabetes, and in enhancement of somatic, hyperplastic, and neoplastic growth indicating an effect(s) on diverse cell types. Its constant ectopic expression results in increased tumor formation (8), qualifying the A^{vy} mutation as a dominant oncogene.

The human homolog of the *agouti* locus (mouse chromosome 2) is located in a syntenic segment on human chromosome 20q (9) and is designated *agouti signaling protein (ASIP)* (10). It is normally expressed at low levels in various tissues but its function remains unknown. No *agouti* locus mutation, equivalent to any found in mice, has been identified in *Homo sapiens.*

Materials and Methods

Animals. Mottled yellow A^{vy}/A and agouti *A/a* mice were produced by mating BALB/cStCrlfC3Hf/Nctr dams with VY/WffC3Hf/Nctr-A^{vy} sires and were maintained in a clean conventional environment. CR mice were housed singly; AL mice were housed two per cage.

Sterilized hardwood chips (P.J. Murphy Forest Products Corp., Rochelle Park, NJ) were used as bedding. Millipore-filtered water was always available. Cages and water bottles were changed weekly. Room temperature was maintained at 22° ± 2°C and relative humidity at 50% ± 5% with 15–17 changes of air/hr. Fluorescent light cycles of 12 hr on (6 AM to 6 PM) and 12 hr off (6 PM to 6 AM) were automatically regulated.

The calorically restricted groups were fed 70% of the calories consumed by an "*ad lib* consumption" group of mice from each category. These groups were fed the *ad libitum* diet 3 weeks earlier. Restriction commenced at 11 weeks of age with 10% restriction for 1 week, 20% for another week, then 30% restriction from 13 weeks onward. All mice were weighed weekly, and groups of 12 mice in each category were sacrificed at 20, 24, 28, and 32 weeks of age. The study was terminated at 68 weeks of age.

Only brain weights were obtained since the other organs had to be frozen in liquid nitrogen immediately after excision for later assays of gene transcription.

This study was approved by the NCTR Institutional Animal Care and Use Committee prior to implementation.

Diets. Diet D12386 A (Table I) was fed *ad libitum* to two separate sets of mice, each including all experimental categories: one set, the "*ad lib* consumption" group, was used to determine the amount of feed to be fed to the CR mice; the second set was used to obtain the control AL data.

To reduce only the caloric intake of the CR mice, Diet 12392 A (Table I) was fed at 71% of the feed consumption of the "*ad lib* consumption" group. Thus the CR mice received 100% of the protein, fat, fiber, salts, and vitamins supplied to the AL mice, but only 70% of the calories.

Table I. Composition of Diets

	AL (D12386A)		CR (D12392A)[a]	
	Percentage composition			
	g %	kcal %	g %	kcal %
Protein	19.2	20.0	27.0	28.6
Carbohydrate	67.3	70.0	54.1	57.2
Fat	4.3	10.0	6.0	14.3
Total:	90.8	100.0	87.1	100.0
kcal/g	3.85		3.78	
	Ingredients			
	g	kcal	g	kcal
Casein	200	800	200	800
L-Cystine	3	12	3	12
Corn starch	475	1900	171	684
Maltodextrin 10	125	500	125	500
Sucrose	100	400	100	400
Cellulose	50	0	50	0
Soybean oil	15	135	15	135
Corn oil	30	270	30	270
Salt mix S10021[b]	10	0	10	0
$CaHPO_4$	13	0	10	0
$CaCo_3$	5.5	0	5.5	0
Potassium citrate monohydrate	16.5	0	16.5	0
Vitamin mix V10001[c]	10	40	10	40
Choline bitartrate	2	0	2	0
Total:	1055	4057	751	2841

[a] For 30% caloric restriction.
[b] Same as AIN-76.
[c] Modified from AIN-76.

Both diets were freshly prepared every 3 months by Research Diets, Inc. (New Brunswick, NJ) and stored at NCTR in a cool room at about 4°C.

Serum Glucose Assays. To assure optimum *in vivo* tissue conditions for identifying diet-regulated genes, the following feeding regimen (11) was used prior to obtaining blood *via* the retro-orbital sinus and subsequent sacrifice of the mice. Feed was removed at 4 PM on the day before sacrifice. At 4 AM of the sacrifice day, 70% of the daily food intake was presented to the animals. At 6 AM the remaining feed was removed from the cages. At 8 AM presacrifice bleeding and sacrifice commenced.

Glucose analyses were performed on the Cobas Mira Plus (Roche Diagnostic Systems, Somerville, NJ) with Roche Diagnostic reagents (hexokinase). The instrument was calibrated according to the manufacturer's recommendations. Two levels of assayed controls were included in each day's analyses as internal controls.

Statistical Methods. Brain weights, body weights, and serum glucose concentrations were analyzed separately. In each case, an initial factorial analysis of variance was conducted, in which all three factors—diet, phenotype, age—were crossed with one another. The SAS procedure GLM (12) was used, with Type III sums of squares selected for testing.

Initially, a completely saturated model was fitted, and effects of all orders were tested. Because the three-factor interaction was not statistically significant at the 10% level, a significance level often used to assess interactions in analysis-of-variance (ANOVA) models, a model with only main effects and two-factor interactions was refitted. Since the results were qualitatively the same for both models, the reduced model was used for statistical testing. Each main-effect test was based on a 5% significance level, and each interaction-effect test on a 10% significance level.

The analyses of body weight and body weight gain utilized the body weights at 51 weeks because of increasing mortality after this age. However, for analyses of brain weights, the data from 68-week-old mice were used.

Results

Body Weights. At 4 weeks of age, the yellow A^{vy}/A mice were already ≈ 14% heavier than the congenic agouti *A/a* mice. Between 4 and 12 weeks of age, the yellow mice gained ≈ 63% in body weight, whereas the agouti mice gained only ≈ 44%. By 12 weeks of age the yellow A^{vy}/A mice were ≈ 24% heavier than the agouti *A/a* siblings. Although not measured in this study, the fat-free dry weights of yellow mice normally exceed those of their nonyellow litter mates (13).

During caloric restriction between 12 and 51 weeks of age, the CR A^{vy}/A *and A/a* mice gained only 16% and 15%, respectively, whereas the AL A^{vy}/A mice gained ≈ 128%, and the AL *A/a* mice gained ≈ 41% (Table II).

As is apparent from Figure 1, the mean body weights of the yellow CR mice resembled those of the agouti AL mice more than those of the agouti CR mice. This may, in part, reflect a greater lean body mass developed by the *CR* A^{vy}/A mice before caloric restriction commenced, as well as the somewhat greater food consumption of the CR A^{vy}/A. Because AL A^{vy}/A mice ate about 20% more than AL *A/a* agouti mice between 16 and 44 weeks of age, the actual feed consumption of the CR A^{vy}/A mice was ≈ 13% greater than that of the CR *A/a* mice. As a result of greater feed consumption, the CR A^{vy}/a mice gained ≈ 11.6 g more than the CR *A/a* mice during this period.

The individual effects of *diet* and *phenotype* and the interaction of *diet* with *phenotype*, on the percentage increase in body weight between 12 and 51 weeks (Table II) were each highly significant statistically ($P < 0.0001$). Thus, the effect of caloric restriction was greater in A^{vy}/A than in *A/a* animals, reducing the weight gain differential between yellow and agouti mice.

Efficiency of Feed Utilization. The body weights of A^{vy}/a AL mice increased considerably more relative to their feed consumption over the course of the study than in the *A/a* AL mice, indicating greater efficiency in calorie utilization by the yellow AL mice (Fig. 2). The feed consumed per gram body weight gain during an active growth period between 16 and 44 weeks illustrates this difference between the yellow and agouti mice: AL A^{vy}/A 8.7 g, *A/a* 30.5 g; CR A^{vy}/A 28.4 g, *A/a* 28.0 g. These data concur with an earlier conclusion "that the expression of the A^{vy} genotype . . . affects the efficiency of food utilization more than the total caloric intake" (14).

For some weeks after initiation of caloric restriction (10% restriction at 11 weeks, 20% at 12 weeks, 30% at 13 weeks), efficiency of nutrient utilization by the CR mice was greater than for the corresponding AL mice (A^{vy}/A 12–16 weeks, *A/a* 12–24 weeks). We have no obvious expla-

Table II. Increase (%Δ) in Body Weights of *Ad Libitum*-Fed (AL) and Calorically Restricted (CR) Yellow A^{vy}/A and Agouti *A/a* (BALB/c × VY)F_1 Female Mice Between 12 Weeks and 51 Weeks of Age

	AL			CR		
	12 wk	51 wk	%Δ	12 wk	51 wk	%Δ
A^{vy}/A	24.9[a] ± 0.7 (36)	56.0 ± 1.6	127.7 ± 6.7	24.2 ± 0.7 (43)	27.5 ± 0.5	16.3 ± 3.0
A/a	20.2 ± 0.3 (35)	28.3 ± 0.6	40.8 ± 2.4	18.4 ± 0.2 (39)	20.9 ± 0.3	14.7 ± 2.2

[a] Mean grams ± S.E. (*n*).

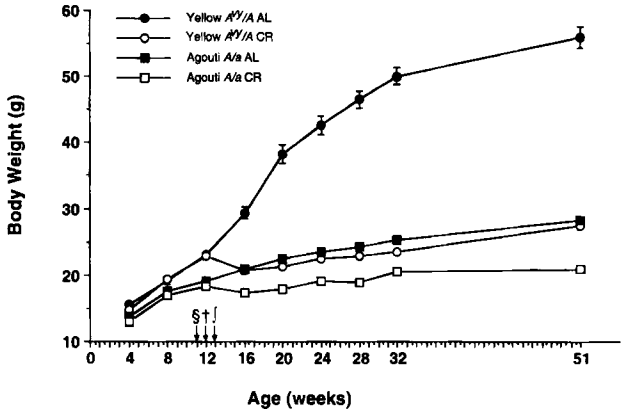

Figure 1. Body weights of *ad libitum* fed (AL) and calorically restricted (CR) yellow A^{vy}/A and agouti *A/a* (BALB/c × VY)F_1 female mice between 4 and 51 weeks of age. As indicated, 10% caloric restriction started at week 11§, increased to 20% at week 12†, and then to 30% at week 13∫ where it was maintained for the remainder of the study. (n = A^{vy}/A AL 36, CR 43; *A/a* AL 35, CR 39)

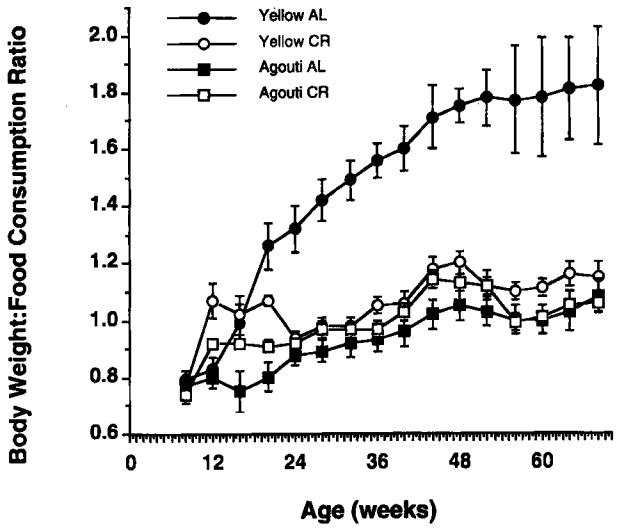

Figure 2. Relation of body weight (g) to amount (g) of feed consumed.

nation for these seemingly paradoxical observations that will have to be resolved by future study.

Brain Weights. Brain weights of CR mice (mean 0.488 g) at 68 weeks of age were significantly lower ($P < 0.0001$) than those of AL mice (mean = 0.511 g). No apparent effect of age on average brain weights was observed. However, as indicated by the group means, there was a highly significant ($P < 0.0001$) *diet* × *phenotype* interaction viz

	AL	*CR*	*% Difference*
A^{vy}/A	0.508	0.497	−2.2
A/a	0.514	0.479	−6.8

It appears that the decrease in brain weights associated with caloric restriction was only one-third as great among the yellow as among the agouti mice.

Serum Glucose Levels. In accord with previous observations, yellow AL mice had uniformly higher serum glucose levels than the agouti AL mice ($P < 0.0001$) (Fig. 3). Caloric restriction significantly ($P < 0.0001$) reduced glucose levels in both yellow and agouti mice; however, glucose levels among A^{vy}/A CR mice were still uniformly higher ($P < 0.0001$) than those among *A/a* CR mice, although the difference (≈ 10%) was not as great as between the A^{vy}/A and *A/a* AL mice (≈ 30%).

Caloric restriction and agouti phenotype each lowered serum glucose concentrations independently ($P < 0.0001$), below those found in AL and A^{vy}/A mice. Caloric restriction resulted in twice as great a decrease in serum glucose concentration in yellow (≈ 32%) as in agouti (≈ 15%) mice. With age, the difference between AL and CR serum glucose levels decreased from ≈ 42% (20 weeks) to ≈ 15% (68 weeks) among the yellow mice. Among the agouti mice a different pattern was observed. A discrepancy of ≈ 16% at 20 weeks between agouti AL and CR mice increased to 20%–23% at 24 and 28 weeks, then decreased to ≈ 7%–8% at 32 and 68 weeks. This genotypic difference in the pattern of age-related differences in serum glucose levels was reflected by a significant ($P < 0.01$) *diet* × *age* × *phenotype* interaction effect.

For parallel graphic presentation of serum glucose concentrations and body weights (Fig. 2), only those mice with available glucose data were used.

Discussion

The data presented here indicate that caloric restriction reduced the weight gain differential between yellow and agouti mice by "normalizing" (i.e., abolishing) the greater efficiency of nutrient utilization by the yellow mice. The only previously published data on caloric restriction of obese yellow mice (pair-fed with lean brown *a/a* congenic siblings from weaning at ≈ 4 weeks of age) were reported in conjunction with induction of pulmonary tumors by 3-methylcholanthrene in "lethal yellow" A^y/a and brown *a/a* (A × YBR)F_1 mice (15).

In the present study, the CR groups were fed 70% of the calories supplied to AL groups from 13 weeks of age until the terminal sacrifice at 68 weeks, except for sample groups sacrificed at 20, 24, 28, and 32 weeks of age.

As is apparent from Figure 1, CR A^{vy}/A mice maintained body weight approximating that of AL *A/a* mice, whereas CR *A/a* mice had mean body weights ≈ 26% lower than AL *A/a* mice.

As suggested by the uniformly higher (≈ 10%) serum glucose concentrations in CR A^{vy}/A mice than in CR *A/a* mice, absence of overt obesity may not completely eliminate insulin resistance in yellow mice if ectopic agouti protein *per se* impacts insulin sensitivity. Present evidence does not establish if the relative proportions of body fat in CR A^{vy}/A and *A/a* were similar. If not, slightly greater fat content in CR A^{vy}/A mice may have induced peripheral insulin resistance sufficient to maintain blood glucose levels slightly higher than in CR *A/a* mice.

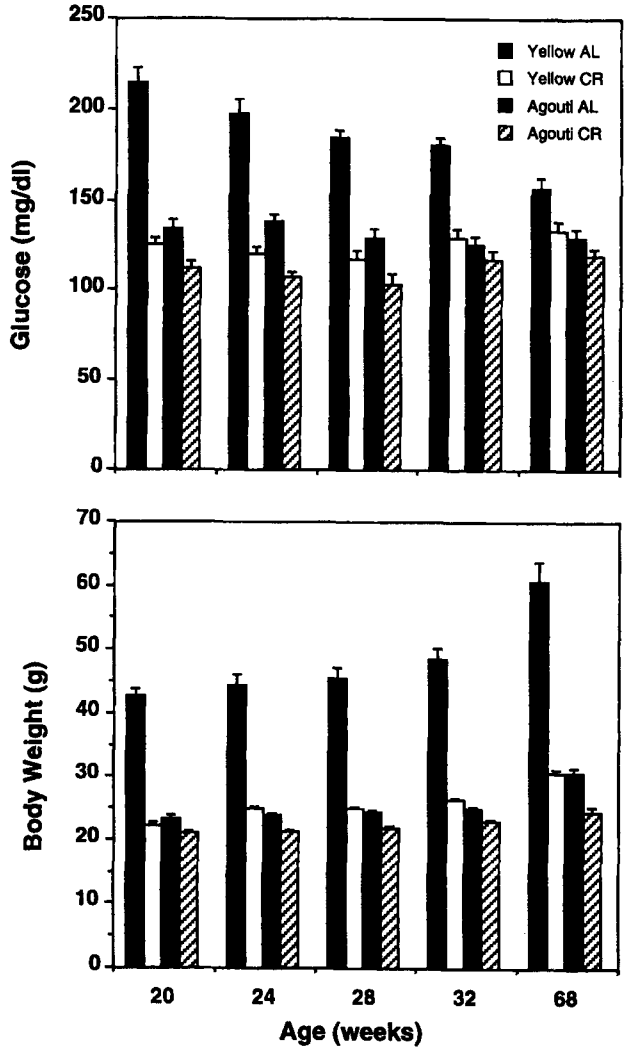

Figure 3. Mean serum glucose concentrations and body weights (± SE) of *ad libitum* fed (AL) and calorically restricted (CR) yellow A^{vy}/A and agouti *A/a* (BALB/c x VY)F_1 female mice between 20 and 68 weeks of age. Body weights only from mice with glucose values.
(*n* = 20 weeks: A^{vy}/A AL 23, CR 23; *A/a* AL 24, CR 21
24 weeks:A^{vy}/A AL 23, CR 23; *A/a* AL 24, CR 23
28 weeks:A^{vy}/A AL 24, CR 21; *A/a* AL 24, CR 23
32 weeks:A^{vy}/A AL 22, CR 23; *A/a* AL 18, CR 20
68 weeks:A^{vy}/A AL 19, CR 33; *A/a* AL 17, CR 34)

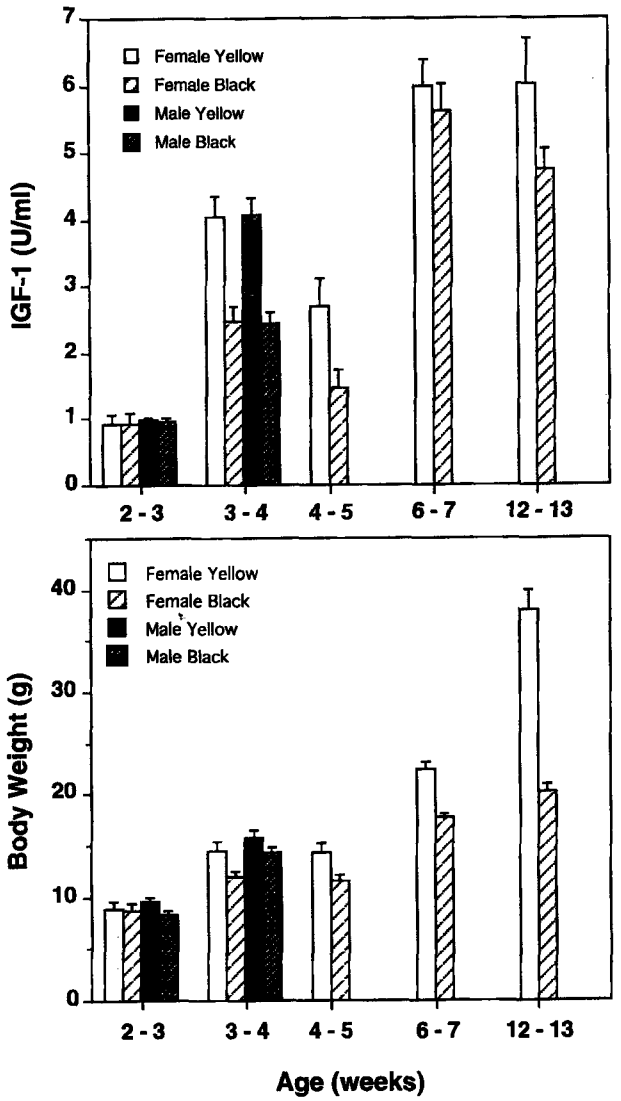

Figure 4. Serum IGF-1 concentrations in yellow A^{vy}/a and black *a/a* Strain VY/WffC3Hf/Nctr-A^{vy} mice between 3 weeks and 3 months of age (data from Ref. 18).
Method: Highly specific heterologous radioimmunoassays for IGF-I, kindly performed by Dr. L.E. Underwood (University of North Carolina), utilized pure IGF-1, from Cohn Fraction IV of human sera, radiolabeled with ^{125}I (19). Pooled human serum was used as the reference standard and was assigned a potency of 1 unit/ml. Dilution curves of the reference standard, as well as those of all mouse sera, paralleled that of pure IGF-1. Intra-assay variation was less than 5% and interassay variation less than 10%.
Number of mice: $n_{females}$: 20.5 ± 1.0 days of age (2–3 weeks) A^{vy}/a 13, *a/a* 10; 27 ± 1.0 d (3–4 weeks) A^{vy}/a 12, *a/a* 12; 30.5 ± 1.5 d (4–5 weeks) A^{vy}/a 8, *a/a* 10; 44.5 ± 1.5 d (6–7 weeks) A^{vy}/a 10, *a/a* 10; 86.5 ± 1.5 d (12–13 weeks) A^{vy}/a 10, *a/a* 10.
n_{males}: 18.5 ± 1.5 d (2–3 weeks) A^{vy}/a 11, *a/a* 12; 26.0 ± 1.0 d (3–4 weeks) A^{vy}/a 11, *a/a* 12.
Sera processing: Sera from females at 4–5, 6–7, and 12–13 weeks of age were obtained and assayed simultaneously. Sera from females at 2–3 and 3–4 weeks were processed simultaneously about six weeks later. All male sera were processed simultaneously about 3 months after the second lot of females.
Results: At 3–4 weeks, yellow males and females had higher IGF-1 levels than black mice of either sex ($P < 0.001$). At 4–5 weeks yellow females had higher IGF-1 concentrations than black females ($P = 0.02$). IGF-1 levels in same genotype females at 3–4 and 4–5 weeks of age were not different ($P > 0.05$).

The greater mean body weight gain (Table II) and larger mean brain weight of CR A^{vy}/A mice, compared to CR *A/a* mice, agree with the hypothesis that ectopic agouti protein, directly or indirectly, exerts stimulatory effects on somatic growth and tumorigenesis in the absence of overt obesity (16).

Even though the specific mechanism is unknown, ectopic overexpression of agouti protein makes yellow mice considerably more efficient than their agouti siblings in converting calories to body mass. In a previous study (14), A^{vy}/A females gained ≈ 14.0 g/kcal; in contrast, *A/a* females gained only ≈ 3.5 g/kcal. In the present study the AL A^{vy}/A mice had a mean feed consumption:body weight gain ratio of ≈ 8.7 between 16 and 44 weeks of age, whereas this ratio for AL *A/a* mice was ≈ 30.5. Under caloric restriction,

greater efficiency of calorie utilization by yellow mice was not apparent as shown by the same feed consumption:body weight gain ratios (≈ 28) for CR A^{vy}/a and CR A/a mice.

Obesity, increased lean body mass, earlier and more rapid tumor formation of yellow mice imply altered metabolic programming as shown by the rate of age-associated decrease in the rate of lipogenesis in yellow mice. In obese A^{vy}/a mice this rate decreased only 9% with age compared with 22% in lean a/a mice and 60% in obese Lep^{ob}/Lep^{ob} mice (reviewed in Ref. 17).

The difference in timing of a major increase in serum IGF-1 concentration in strain VY mice (18) shows altered developmental programming with implied effects on growth. The increase in both black a/a and yellow A^{vy}/a was observed beginning at 3 weeks of age (Fig. 4); however, until ≈ 5 weeks it was twice as great in the yellow mice (339% in females, 322% in males) as in the black mice (167% in females, 154% in males). In contrast, between 5 and 13 weeks of age serum IGF-1 levels increased 164% in the black females but only 78% in the yellow females. Thus, whereas the overall increase in circulating IGF-1 was similar in both genotypes, this presumptively mitogenic stimulus was twice as great earlier (between ≈ 3 and 5 weeks of age) in yellow mice than in agouti mice. This suggests that such a transiently higher circulating IGF-1 level in yellow mice may alter their normal metabolic programming and contribute to the greater lean body mass observed in older A^{vy}/A mice and may also be associated with their greater metabolic efficiency.

The data presented confirm effects of ectopic agouti protein on metabolic efficiency and somatic growth in yellow A^{vy}/A mice. They suggest that the protein alters developmental/metabolic programming in ectopic sites, possibly during prenatal and neonatal development, resulting in the multiple diverse phenotypic effects of the viable yellow mutation. Identification of the specific cellular processes modulated by the agouti protein in these ectopic sites remains to be accomplished.

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