A BRIEF COMMUNICATION

Agouti Signaling Protein Stimulates Cell Division in "Viable Yellow" (A^{vy}/a) Mouse Liver

GEORGE L. WOLFF,*,[1] J. STEVEN STANLEY,* MATTHEW E. FERGUSON,* PIPPA M. SIMPSON,† MARTIN J. J. RONIS,*,‡ AND THOMAS M. BADGER*,†,§,[1]

Arkansas Children's Nutrition Center; †Department of Pediatrics; ‡Department of Pharmacology/Toxicology; and §Department of Physiology/Biophysics, University of Arkansas for Medical Sciences, Little Rock, Arkansas 72202

Enhanced linear growth, hyperplasia, and tumorigenesis are well-known characteristics of "viable yellow" agouti $A^{vy}/-$ mice (Wolff GL, Roberts DW, Mountjoy KG. Physiol Genomics 1:151–163, 1999); however, the functional basis for this aspect of the phenotype is unknown. In the present study, we ascertained whether agouti signaling protein (ASIP) levels in A^{vy}/a or a/a livers are associated with hepatocyte proliferation as a possible factor in promotion of hepatocellular tumor formation. Proliferating cell nuclear antigen (PCNA) assays and quantitative real-time reverse transcriptase polymerase chain reaction assays were performed on liver samples from mottled yellow A^{vy}/a, pseudoagouti A^{vy}/a, and black a/a VY mice to determine mitotic indices and expression levels of A^{vy} and a in relation to the expression level of the housekeeping gene *hprt*. We found that ASIP levels were ~100-fold higher in yellow than in pseudoagouti or black mice and that the proportion of PCNA-positive hepatocytes was greater ($P < 0.001$) in yellow than in pseudoagouti or black mice. Exp Biol Med 232:1326–1329, 2007

Key words: agouti signaling protein; cell division; pseudoagouti mice

Introduction

Elevated levels of agouti signaling protein (ASIP) in the tissues of yellow agouti obese A^{vy}/a mice result from continuous transcription of the *agouti* gene induced by a cryptic promoter in the intracisternal A particle (IAP) retrotransposon inserted in noncoding exon 2 of the *agouti* locus (1). In wild-type "white-bellied agouti" $A^{w}/-$ mice, ASIP is synthesized by the *agouti* locus only during the first 4–7 days of hair growth when the subapical yellow band, characteristic of the agouti coat color pattern, is formed. Because of methylation of the CpG islands on the cryptic IAP promoter in pseudoagouti $A^{vy}/-$ mice, ASIP synthesis is regulated as in $A^{w}/-$ mice. Thus, pseudoagouti $A^{vy}/-$ mice, epigenetically different from but genetically identical to mottled yellow mice, exhibit the wild-type agouti coat color pattern and remain lean, normoglycemic, and normoinsulinemic. The loss-of-function nonagouti *a* mutation at the *agouti* locus results in the synthesis of an inactive ASIP in black a/a mice (2).

Binding of the inverse agonist ASIP by melanocortin receptor-1 (MC1-R) prevents binding of the normal MC1-R ligand, α-melanocyte stimulating hormone (αMSH). Thus, eumelanogenesis is prevented, and pheomelanogenesis occurs during the whole hair growth cycle (1). The obesity-associated effects, adult-onset male and female obesity, and male hyperinsulinemia characteristic of mottled yellow $A^{vy}/-$ mice (3, 4), on the other hand, result from binding of ASIP to melanocortin receptor-4 (MC4-R) neurons in the hypothalamus; thus, MC4-R signaling is inactivated (5). In the presence of low normal levels of

This work was funded in part by USDA ARS grant 6251-51000-005-02S.

[1] To whom correspondence should be addressed at 2802 Millbrook Road, Little Rock, AR 72227-3038. E-mail: gwolffar@prodigy.net (G.L.W.), or Children's Nutrition Center, 1120 Marshall Street, Little Rock, AR 72202. E-mail; badgerthomasm@uams.edu (T.M.B.)

Received April 12, 2007.
Accepted June 23, 2007.

DOI: 10.3181/0704-BC-95
1535-3702/07/23210-1326$15.00

ASIP, MC4-R signaling is regulated by its agonist agouti-related peptide (AGRP). Like lethal yellow A^y/a mice (6), *MC4-R* knock-out mice exhibit increased body length and obesity (7). No information on the possible effects of the absence of MC4-R signaling on hyperplasia or tumorigenesis is available.

Hyperplasia, preceding neoplastic transformation and tumorigenesis, in diverse tissues is more prevalent in yellow agouti A^{vy}/a and A^y/a mice than in their sibling black a/a or agouti $A/-$ siblings (5). For example, Warbritton *et al.* (8) reported β-cell pancreatic hyperplasia in 21-day-old pre-obese yellow A^{vy}/a mice. Body length and fat-free dry mass of yellow agouti mice are also greater than those of sibling black mice (6). Whether the tendencies to increased body size, hyperplasia, and tumorigenesis are associated with mild hyperinsulinemia of yellow mice rather than with elevated ASIP levels has been unclear.

Previously we found the mitotic index in male mottled yellow A^{vy}/a mouse livers to be 4-fold greater than that in male a/a livers (9). In the absence of MC1-R and MC4-R (10), ASIP generated in mouse adipose tissue and liver, respectively, by tissue-specific transgenes upregulates adipocyte transcription factors (11) or resulted in an increased prevalence of hepatic adenocarcinoma in response to diethylnitrosamine (DEN) (12). ASIP also upregulates the transcription of several melanogenic and other genes in "melan-a" melanocyte cell line cultures and newborn mouse skin, whereas αMSH downregulates transcription of these genes (13). Pseudoagouti is an A^{vy}/a phenotype in which CpG islands in the long terminal repeat (LTR) of the cryptic IAP promoter of the *agouti* gene have been hypermethylated (14); thus, the IAP promoter is silenced, and the hair-cycle promoters in exons 1B and 1C are enabled to regulate *agouti* gene transcription. Consequently, in pseudoagouti A^{vy}/a mice, ASIP is synthesized in the hair follicle cells only during days 4–7 of the hair growth cycle. Using a reverse transcriptase polymerase chain reaction (RT-PCR) assay, Duhl *et al.* (1) found that ASIP was present in all examined tissues of pseudoagouti mice. To quantify this observation in liver, normalized ASIP levels in pseudoagouti, mottled yellow, and black VY mouse livers were assayed by quantitative real-time RT-PCR in the present study.

Materials and Methods

Animals. A breeding colony derived from the highly inbred (>200 generations) VY/WffC3Hf/Nctr-A^{vy} colony at the National Center for Toxicological Research/FDA, Jefferson, AR, was initiated at the Arkansas Children's Nutrition Center. Matings of black a/a dams by mottled yellow and pseudoagouti A^{vy}/a sires produced A^{vy}/a and a/a offspring for this study. At weaning (about 4 weeks of age) transponder chips were implanted interscapularly in each weanling for identification. Two to 5 mice were housed in covered polycarbonate cages in a facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

Because of a paucity of clear yellow (CY) progeny, it was necessary to assay hepatic ASIP levels and proliferating cell nuclear antigen (PCNA) prevalence also in slightly mottled yellow (SMY), mottled yellow (MY), and heavily mottled yellow (HMY) mice. Body and fat pad weights of these mouse phenotypes will be presented elsewhere. Cages were changed weekly by using Tek-Fresh bedding (Harlan Teklad, Indianapolis, IN). Mice had *ad libitum* access to AIN93G diet and water. A 6 AM to 6 PM light/dark cycle, a temperature of ~22°C, and a humidity of ~48% were automatically maintained. This study was approved by the University of Arkansas for Medical Sciences Institutional Animal Care and Use Committee.

Quantitative Real-Time RT-PCR. Total RNA was isolated from ~100 mg of mouse liver by using TRIzol reagent (Invitrogen, Carlsbad, CA) and the RNeasy Mini Kit (QIAGEN, Valencia, CA). DNA was removed by digestion with RNase-free DNase (QIAGEN). The integrity of the RNA was examined by using the Agilent Bioanalyzer System (Agilent Biotechnologies, Palo Alto, CA). One microgram of total RNA from each mouse liver was reverse-transcribed by using the iSript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). SYBR Green real-time PCR was performed by using the MyiQ real-time PCR machine (Bio-Rad Laboratories). Thermal cycling conditions included preincubation at 95°C for 2 mins followed by 40 PCR cycles at 95°C for 30 secs, and 60°C for 1 min. The level of RNA expression was expressed as a ratio relative to the level of RNA expression of the housekeeping gene *hprt*. Primers used to determine relative concentrations of ASIP were the following: *hprt* forward, 5′-AGG-ACC-TCT-CGA-AGT-GTT-GG-3′; *hprt* reverse, 5′-CAG-ATT-CAA-CTT-GCG-CTC-AT-3′; ASIP agouti forward: GCTGATGCGGAATAGAGTCA; ASIP agouti reverse, TTCTTGTTCAGTGCCACGAT.

PCNA Assays. PCNA assays were performed as previously described (9) on the same mouse livers used in the real-time RT-PCR assays. PCNA is a marker of cells in the early G1 phase and S phase of the cell cycle. The mitotic index was calculated by dividing the number of stained hepatocytes by the total number of hepatocytes in the tissue section.

Statistical Analyses. Box plots and summary statistics were used to assess the distribution of variables. First, analysis of variance with sex, phenotype group, and interactions was done. A Tukey HSD test was used to adjust for *post hoc* multiple comparisons. Because there were outliers, a nonparametric Kruskal-Wallis analysis of variance was also used to compare groups. Mann-Whitney tests were used for comparisons between two groups. With few exceptions, the two methods agreed. Results are cited for the nonparametric test. All tests were two-tailed. The P values were adjusted for multiple comparisons by using a Holms step-down procedure (15).

Table 1. ASIP Levels[a] and % PCNA-Positive Hepatocytes (G_1+S+G_2+M) in Yellow/ Mottled Yellow and Pseudoagouti A^{vy}/a and Black a/a Strain VY Mouse Liver

	ASIP		% PCNA-positive hepatocytes		Hepatic carcinoma[c]
	Mean	Range	Mean	Range	(%)
Males					
Yellow/mottled	0.1747	0.11–0.27 (12)	1.5669	0.39–3.36 (12)	—
Pseudoagouti	0.0018	0.00–0.01 (11)	1.2833	0.66–2.49 (9)	—
Black	0.0001[b]	0.00–0.01 (14)	0.8138	0.54–1.45 (15)	—
Females					
Yellow/mottled	0.2349	0.00–0.33 (15)	1.5083	0.50–3.07 (15)	13
Pseudoagouti	0.0023	0.00–0.02 (9)	0.9844	0.62–1.84 (8)	2
Black	0.0009[b]	0.00–0.01 (16)	1.1132	0.46–1.63 (15)	3

[a] Relative to expression levels of *hprt*.
[b] Inactive ASIP.
[c] Reference 19.

Results and Discussion

In VY/Wf mouse liver ASIP levels were ~100 fold higher in obese mottled yellow A^{vy}/a mice than in lean pseudoagouti A^{vy}/a and black a/a mice ($P < 0.001$) (Table 1). The levels of active ASIP in pseudoagouti A^{vy}/a males were ~18-fold higher than levels of inactive ASIP in black a/a males. ASIP levels in pseudoagouti females were ~2.5-fold higher than inactive ASIP levels in black females. Although the loss-of-function mutation *a* results in inactive ASIP in black mice, the low level of active ASIP in pseudoagouti mice results from DNA methylation-induced inhibition of *agouti* gene transcription (14).

The proportion of mitotically active hepatocytes in male mottled yellow and pseudoagouti A^{vy}/a mouse livers was significantly greater ($P < 0.05$) than that in black a/a males (Table 1). Male pseudoagouti mouse livers did not differ significantly in mitotic activity from those of the yellow mice ($P > 0.05$). Although gender had no effects on active ASIP levels in mice, inactive ASIP levels appeared to be somewhat higher among female a/a mice than among a/a males (Table 1). No obvious explanation for this gender difference is apparent. Promotion of hyperplastic alveolar nodule (HAN) formation (16) and earlier appearance of mammary adenocarcinomas (17) as well as formation of hepatic adenocarcinomas (18, 19), adenomas, and lung tumors (19) has been associated with elevated ASIP.

Kuklin *et al*. (12) generated transgenic mice in which the albumin promoter directed liver-specific expression of the wild-type murine *agouti* cDNA. Although ASIP was expressed in the liver at a higher level than in A^{vy}/a mice, the transgenic mice did not become obese and remained normoglycemic and normoinsulinemic. However, in response to a single injection of DEN, a greater number of hepatocellular adenocarcinomas developed in the transgenic mice than in the control mice. This finding suggests, as does the response to lindane (1), that elevated ASIP acts primarily as a tumor promoter rather than as an initiator.

Although some of the nonpigment-related effects of ASIP (e.g., hyperphagia and obesity) are mediated by its binding to MC4-R, ASIP also affects tissues where MC4-R is absent: the liver (12) and adipose tissue (11). For example, ASIP promoted a greater prevalence of hepatocellular adenocarcinoma (Table 1; Ref. 19). The upregulation by ASIP of adipocyte transcription factors (11) and ASIP stimulation of cellular proliferation in mouse liver (Table 1) suggest that ASIP may also exert its growth stimulatory effects in the body.

The seeming discrepancy between the ~100-fold difference in ASIP level and the approximately 22% (males), 53% (females) differences between the median mitotic indices of the yellow and pseudoagouti mice suggests that ASIP may stimulate hepatocyte mitosis indirectly, possibly by upregulation of transcription factors regulating gene expression in proliferative pathways. A possible candidate is the T-cell transcription factor-4 (TCF-4), previously designated ITF2, that is upregulated by ASIP and downregulated by α-MSH in "melan-a" melanocyte cell line cultures (20). TCF-4 is a component of the Wnt signaling pathway and, when in a complex with β-catenin, acts as a transcription factor for genes involved in cell proliferation and carcinogenesis (e.g., *cyclin D1*, c-*jun*, and c-*myc* [21]). Higher levels of TCF-4 in A^{vy}/a livers than in a/a livers were detected in the present study (unpublished data).

We thank S. R. Till for performing the PCNA assays and E. Kozlowska for the real-time RT-PCR assays.

1. Duhl DMJ, Vrieling H, Miller KA, Wolff GL, Barsh GS. Neomorphic agouti mutations in obese yellow mice. Nat Genet 8:59–65, 1994.
2. Bultman SJ, Klebig ML, Michaud EJ, Sweet HO, Davisson MT, Woychik RP. Molecular analysis of reverse mutations from nonagouti (a) to black-and-tan (a^t) and white-bellied agouti (A^w) reveals alternative forms of agouti transcripts. Genes Dev 8:481–490, 1994.
3. Frigeri LG, Wolff GL, Robel G. Impairment of glucose tolerance in yellow (A^{vy}/A) (BALB/c x VY)F-1 hybrid mice by hyperglycemic

peptides from human pituitary glands. Endocrinology 113:2097–2105, 1983.
4. Wolff GL, Greenman DL, Frigeri LG, Morrissey RL, Suber RL, Felton RP. Diabetogenic response to streptozotocin varies among obese yellow and among lean agouti (BALB/c x VY)F_1 hybrid mice. Proc Soc Exp Biol Med 193:155–163, 1990.
5. Wolff GL, Roberts DW, Mountjoy KG. Physiological consequences of ectopic agouti gene expression: the yellow obese mouse syndrome. Physiol Genomics 1:151–163, 1999.
6. Wolff GL. Growth of inbred yellow ($A^y a$) and non-yellow (*aa*) mice in parabiosis. Genetics 48:1041–1058, 1963.
7. Huszar D, Lynch CA, Fairchild-Huntress V, Dunmore JH, Fang Q, Berkemeier LR, Gu W, Kesterson RA, Boston BA, Cone RD, Smith FJ, Campfield LA, Burn P, Lee F. Targeted disruption of the melanocortin-4 receptor results in obesity in mice. Cell 88:131–141, 1997.
8. Warbritton A, Gill AM, Yen TT, Bucci T, Wolff GL. Pancreatic islet cells in pre-obese yellow A^{vy}/- mice: relation to adult hyperinsulinemia and obesity. Proc Soc Exp Biol Med 206:145–151, 1994.
9. Whittaker P, Dunkel VC, Bucci TJ, Kusewitt DF, Thurman JD, Warbritton A, Wolff GL. Genome-linked toxic responses to dietary iron overload. Toxicol Pathol 25:556–564, 1997.
10. Mountjoy KG, Wu CSJ, Dumont LM, Wild JM. Melanocortin-4 receptor messenger ribonucleic acid expression in cardiorespiratory, musculoskeletal, and integumentary systems. Endocrinology 144: 5488–5496, 2003.
11. Mynatt RL, Stephens JM. Agouti regulates adipocyte transcription factors. Am J Physiol Cell Physiol 280:C954–C961, 2001.
12. Kuklin AI, Mynatt RL, Klebig ML, Kieffer LL, Wilkison WO, Woychik RP, Michaud EJ. Liver-specific expression of the agouti gene in transgenic mice promotes liver carcinogenesis in the absence of obesity and diabetes. Mol Cancer 3:17, 2004.
13. Furumura M, Sakai C, Potterf SB, Vieira WD, Barsh GS, Hearing V. Characterization of genes modulated during pheomelanogenesis using differential display. Proc Natl Acad Sci U S A 95:7374–7378, 1998.
14. Cooney CA, Dave AA, Wolff GL. Maternal methyl supplements in mice affect epigenetic variation and DNA methylation of offspring. J Nutr 132:2393S–2400S, 2002.
15. Westfall PH, Tobias RD, Rom D, Wolfinger RD, Hochberg Y. Multiple Comparisons and Multiple Tests Using the SAS System. Cary, North Carolina: SAS Institute, pp30, 1999.
16. Wolff GL, Medina D, Umholtz RL. Manifestation of hyperplastic alveolar nodules and mammary tumors in "viable yellow" and non-yellow mice. J Natl Cancer Inst 63:781–785, 1979.
17. Wolff GL, Kodell RL, Cameron AM, Medina D. Accelerated appearance of chemically induced mammary carcinomas in obese yellow (A^{vy}/A) (BALB/c x VY) F_1 hybrid mice. J Toxicol Environ Health 10:131–142, 1982.
18. Wolff GL, Pitot HC. Variation of hepatic malic enzyme capacity with hepatoma susceptibility in mice of different genotypes. Cancer Res 32: 1861–1863, 1972.
19. Wolff GL, Roberts DW, Morrissey RL, Greenman DL, Allen RR, Campbell WL, Bergman H, Nesnow S, Frith CH. Tumorigenic responses to lindane in mice: potentiation by a dominant mutation. Carcinogenesis 8:1889–1897, 1987.
20. Furumura M, Potterf SB, Toyofuku K, Matsunaga J, Muller J, Hearing VJ. Involvement of ITF2 in the transcriptional regulation of melanogenic genes. J Biol Chem 276:28147–28154, 2001.
21. Polakis P. Wnt signaling and cancer. Genes Dev 14:1837–1851, 2000.

ERRATA

In the Brief Communication, ‘‘Agouti Signaling Protein Stimulates Cell Division in ‘Viable Yellow’ (A^{vy}/a) Mouse Liver’’ by George L. Wolff, J. Steven Stanley, Matthew E. Ferguson, Pippa M. Simpson, Martin J. J. Ronis, and Thomas M. Badger, which appeared in *Experimental Biology and Medicine* 232(10): 1326–1329, there was an error in the third sentence of the fourth paragraph of the ‘‘Introduction.’’ The sentence should have stated, ‘‘ASIP also upregulates the transcription of several non-melanogenic genes in ‘‘melan-a’’ melanocyte cell line cultures and newborn mouse skin, while αMSH downregulates transcription of these genes (13).’’ The authors regret this error.

In the table of contents in the January 2008 issue of *Experimental Biology and Medicine* (Volume 233, Number 1), the name of one of the authors in the article, ‘‘Chemoprevention of Arylamine-Induced Colorectal Aberrant Crypts,’’ was misspelled. The fourth author’s name should have been D.W. Hein. The author’s name is spelled correctly in the article. The printer apologizes for the error.