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Agouti regulation of intracellular calcium: Role in the insulin resistance of viable yellow mice

(intracellular free calcium/A^{vy} allele)

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

The mouse agouti gene is normally involved in regulating the production of pigment granules that give rise to the wild-type coat color, which consists of black hairs with a subapical band of yellow (1, 2). Several dominant mutations at the agouti locus, most notably lethal yellow (A^y) and viable yellow (A^{vy}), cause mice to develop a predominantly yellow coat color and to become obese, insulin resistant, and hyperinsulinemic with age (reviewed in refs. 3–5). These mutants are collectively often called "yellow obese" mutants.

The agouti gene has been cloned and shown to encode a 131-amino acid protein with a consensus signal peptide (6, 7). Agouti protein is normally expressed in the skin during hair growth (6). Bultman *et al.* (6) discussed a model for the function of the agouti protein that was recently validated at the molecular level (8). Agouti functions in a paracrine manner to regulate the differential production of melanin pigments by the melanocyte (1, 2). Normally, α melanocyte-stimulating hormone (α-MSH) binds to its receptor on the melanocyte, activates adenylate cyclase, and thereby causes an increase in intracellular cAMP levels, which stimulates production of eumelanin (black pigment) (9). However, when agouti is present within the hair follicle, it appears to block the ability of α-MSH to activate its receptor, thereby inhibiting cAMP production and causing a shift from eumelanin to phaeomelanin (yellow pigment) production (8).

While it is becoming increasingly clear how the agouti protein functions in the hair follicle, much less is known about how the agouti gene causes obesity and insulin resistance in the yellow mutants. Molecular analysis of A^y (6, 10, 11), A^{vy} (3), and a new dominant allele of agouti called A^{iapy} (12) revealed in all cases that the agouti gene, which normally is expressed in the developing hair follicle, has been modified in a manner that causes it to be expressed in most, if not all, tissues of the animal. Although the agouti gene is being expressed with an altered tissue distribution, it appears to have retained the ability to produce a normal agouti protein. Agouti is a secreted molecule; however, agouti appears to function in a localized manner (2). Accordingly, the site of synthesis of the agouti protein is an important factor to consider in evaluating its biological activity in the animal. In this regard, it is presently unclear if it is the ubiquitous expression of agouti *per se* or perhaps the ectopic expression of agouti in a specific tissue that is directly responsible for the development of obesity and the other dominant pleiotropic effects.

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

MATERIALS AND METHODS

Animals. C57BL/6J-A^{vy} mice were purchased from The Jackson Laboratory and maintained at the Oak Ridge National Laboratory (Oak Ridge, TN) by mating A^{vy}/a mice to a/a nonagouti black siblings. Experiments were conducted on 3- to 5-month-old male and female viable yellow (A^{vy}/a) mice exhibiting either pseudoagouti, mottled, or yellow coat colors (see *Results*) and were compared with age-matched nonagouti black (a/a) mice.

Preparation of Isolated Skeletal Myocytes. Isolated soleus and gastrocnemius myocytes were prepared essentially as described by Beam and Knudson (20). Briefly, tissue was

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Abbreviations: $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; α-MSH, α melanocyte-stimulating hormone.

isolated from animals following an overnight fast and gently teased apart along the longitudinal axis. The tissue was then incubated at 37°C for 40 min in a Hepes-buffered salt solution (HBSS; 138 mM NaCl/1.8 mM $CaCl_2$/0.8 mM $MgSO_4$/0.9 mM NaH_2PO_4/4 mM $NaHCO_3$/25 mM glucose/6 mM glutamine/20 mM Hepes/0.5% bovine serum albumin) containing type I collagenase at 2 mg/ml. After filtration and centrifugation, the cell pellets were resuspended in HBSS for measurement of $[Ca^{2+}]_i$.

Preparation of Cultured L6 Myocytes. L6 skeletal muscle myocytes were purchased from American Type Culture Collection (Rockville, MD) in passage 4. Cells (1.8×10^6) were plated in a 150-cm^2 flask containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum, 5% fetal bovine serum, 50 units of penicillin per ml, 5 μg of streptomycin per ml, and 10% glucose and were maintained in an atmosphere containing 5% CO_2 and 100% humidity. For sequential passage, nonconfluent cells were rinsed with a Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution (Sigma) and treated with trypsin at 0.5 mg/ml for 2 min. Released cells were recovered by centrifugation. For $[Ca^{2+}]_i$ determination, cells were trypsinized and resuspended in HBSS at a density of $\approx 10^6$ cells per ml.

Expression of Murine Agouti cDNA. A 707-bp *Eco*RV/*Pst* I fragment of the full-length agouti cDNA (6) was subcloned into a *Sma* I/*Pst* I site in the baculoviral expression vector pVL1393 (PharMingen), and the construct was verified by sequencing. This construct was then packaged and titered by standard methods (21). *Trichiplusia ni* cells were then infected at a multiplicity of infection of 2, and the medium was collected 48 hr after infection. This medium was then filtered with a 5-kDa cutoff Sartorius filter and used directly ("agouti-conditioned" medium). Controls consisted of medium alone and medium collected 48 hr after infection from *T. ni* cells infected with the wild-type baculovirus ("control medium").

Rabbit anti-peptide antibodies were generated against a fragment of murine agouti comprising the predicted amino acid residues from position 25 to position 40 (6). Samples of control and conditioned medium were electrophoresed on a 4–20% SDS/PAGE gel and blotted to nitrocellulose. Immunoblots (Western blots) were performed with the agouti anti-peptide antibody in 50 mM Tris, pH 7.5/150 mM NaCl/3% bovine serum albumin (fraction 5, Sigma) at a 1:500 dilution. The second antibody was goat anti-rabbit IgG conjugated to alkaline phosphatase at 1:3000.

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

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

$^{45}Ca^{2+}$ Efflux and Influx. Soleus $^{45}Ca^{2+}$ efflux and influx were determined by using slight modifications of methods previously described (28). For efflux, the soleus muscle was loaded with $^{45}Ca^{2+}$ by incubation in a physiological salt solution (PSS) containing 1 μCi (37 kBq) of $^{45}Ca^{2+}$/ml while being gassed with 95% CO_2/5% O_2 at 37°C. The washout of radioactivity into unlabeled medium was then followed for 90 min. $^{45}Ca^{2+}$ efflux was expressed as a percentage of the original $^{45}Ca^{2+}$ load remaining in the tissue at each time point (28), and the Ca^{2+} efflux rate constant was then calculated from the $^{45}Ca^{2+}$ efflux curve. To determine Ca^{2+} influx, soleus segments were equilibrated in PSS for 10 min at 37°C while being gassed with 95% CO_2/5% O_2. They were then transferred to PSS containing 1 μCi of $^{45}Ca^{2+}$/ml for 2–10 min to measure the rate of Ca^{2+} influx.

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

RESULTS

To evaluate the effect of the ectopic expression of the agouti gene on $[Ca^{2+}]_i$, we chose to use mice carrying the A^{vy} mutant allele as our model animals. A^{vy}/a mice are especially well suited for these experiments because the agouti gene is ectopically expressed at various levels in individual animals that carry the mutant sequences, and the level of agouti expression correlates with the degree to which the animals express the mutant phenotype (Fig. 1). For example, $A^{vy}/-$ mice with high or moderate levels of ectopic agouti expression have completely yellow fur or are mottled with patches of agouti-like hair mixed with totally yellow hair, respectively; these animals have a high propensity to develop the obesity and hyperinsulinemia traits. On the other hand, mice that ectopically express agouti at very low levels have a coat color that is similar to the wild-type agouti color and are referred to as pseudoagouti. Pseudoagouti mice have normal body weights and are not hyperinsulinemic. Although there is a direct correlation between the amount of yellow pigment in the coats of $A^{vy}/-$ mice and the level of agouti expression in many tissues of the body (Fig. 1), mice with even small amounts of yellow pigment in their coats may become obese and hyperinsulinemic, suggesting that there may a threshold level at which agouti exerts these

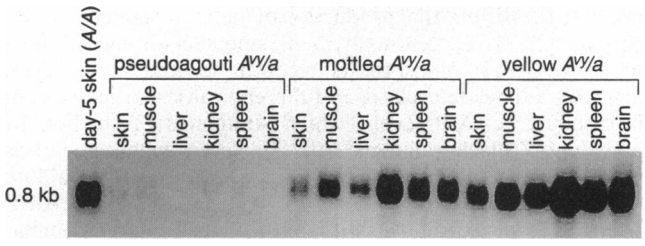

FIG. 1. Northern blot analysis of agouti locus expression in various tissues from adult viable yellow (A^{vy}/a) mice exhibiting either a completely pseudoagouti, moderately mottled (pseudoagouti plus yellow mix), or solid yellow coat color. Neonate skin from a day 5 wild-type agouti (A/A) mouse was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

effects. Therefore, the effect of agouti protein on $[Ca^{2+}]_i$ and insulin resistance can be studied in sibling $A^{vy}/-$ mice that differ only in their level of agouti expression, and their coat colors provide a general indication of the level of ectopic agouti expression and their propensity to become obese and hyperinsulinemic.

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

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

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

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

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

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

*Significantly different from nonagouti black ($P < 0.01$).
†Units for the efflux and influx rates are as follows: efflux, min^{-1}; influx, cpm/ng of protein/min.

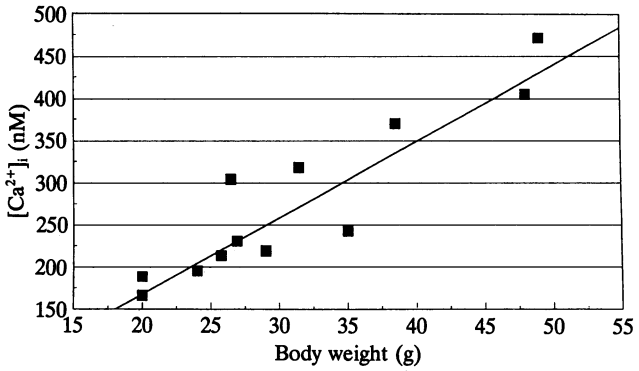

FIG. 2. Relationship between body weight and $[Ca^{2+}]_i$ in freshly isolated soleus myocytes from viable yellow mice with either a pseudoagouti, mottled or yellow coat color. There is a significant correlation ($r = 0.91$, $P < 0.01$; $n = 18$) between $[Ca^{2+}]_i$ and body weight.

antagonize the ability of α-MSH to stimulate cAMP production in B16f10 melanoma cells (8).

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

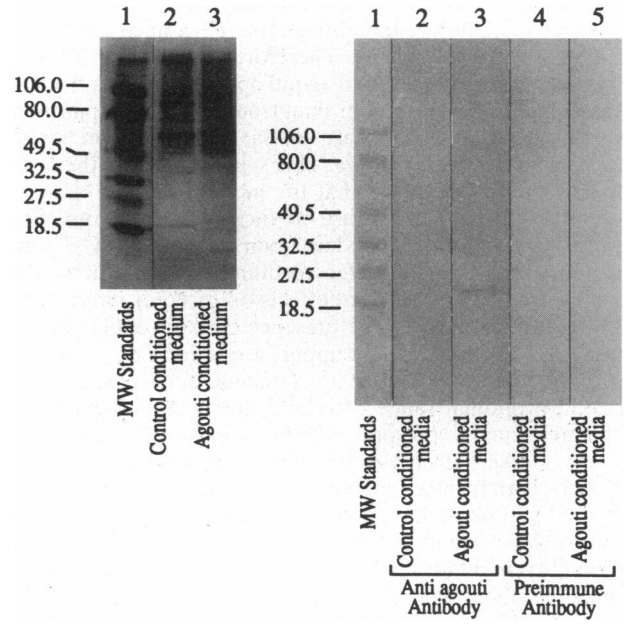

FIG. 3. Expression of the murine agouti peptide. Ten micrograms of medium protein from *T. ni* cells 48 hr after infection of either a wild-type baculovirus or the agouti expression baculovirus was loaded onto a 4–20% SDS/PAGE gel (NOVEX, San Diego) and silver-stained. A duplicate gel was transferred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

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

Treatment*	Medium	
	Control	Agouti conditioned
Ca^{2+}-containing	84.8 ± 3.3	123.2 ± 4.8†
Ca^{2+}-free	88.7 ± 4.0	95.7 ± 2.7

*n = 8 per group.
†$P < 0.01$.

dependent upon extracellular Ca^{2+}, as no such increase was found in Ca^{2+}-free medium (Table 2).

DISCUSSION

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

Although the A^{vy}/a mice exhibited a decrease in insulin-stimulated soleus Ca^{2+} efflux, there was no decrease in basal efflux. This is consistent with our previous observations in rat aortic smooth muscle, in which impaired Ca^{2+}-ATPase-mediated Ca^{2+} efflux was a result rather than a cause of insulin resistance (23, 30–32). In contrast, the A^{vy}/a mice did exhibit an increase in Ca^{2+} influx. Therefore, increased $[Ca^{2+}]_i$ in mice carrying the A^{vy} allele of agouti appears to result from an increase in Ca^{2+} influx rather than from either an impairment in Ca^{2+} efflux or an increase in Ca^{2+} release from sarco/endoplasmic reticulum stores. This suggestion is further supported by the observation that the increase in $[Ca^{2+}]_i$ in L6 skeletal myocytes in response to incubation in agouti-conditioned medium was dependent upon extracellular Ca^{2+} and did not occur in a Ca^{2+}-free medium. However, it is also possible that agouti-mediated effects on receptor interactions may be dependent upon the presence of extracellular Ca^{2+}.

Several lines of evidence support a role for Ca^{2+} in modulating tissue insulin sensitivity. Draznin *et al.* (13) demonstrated an optimal range of $[Ca^{2+}]_i$ for maximizing insulin-stimulated glucose transport, with elevations beyond this range causing marked decreases in adipocyte insulin sensitivity. Similarly, data from a number of studies indicate that increasing $[Ca^{2+}]_i$ in isolated adipocytes results in significant inhibition of insulin-stimulated glucose transport (17–19) and oxidation (14). In addition, Ca^{2+} entry blockade in obese elderly humans resulted in significant increases in peripheral insulin sensitivity (15, 16). Finally, Resnick *et al.* (33) reported that obese patients exhibited a 41% increase in $[Ca^{2+}]_i$ compared with their lean counterparts. Moreover, there was a significant positive correlation between erythrocyte $[Ca^{2+}]_i$ and body mass index in lean and obese subjects, similar to the correlation between soleus muscle $[Ca^{2+}]_i$ and body weight observed in the present study (Fig. 2).

While the relationship between $[Ca^{2+}]_i$ and insulin signal transduction is not well understood, it appears that elevations in $[Ca^{2+}]_i$ may, in part, result in insulin resistance by affecting the phosphorylation of glucose transporter type 4 (Glut4) and other insulin-sensitive substrates within the cell (17–19). Glut4 is the primary insulin-responsive glucose transporter in the cell, and its activity is regulated by serine phosphorylation (34). Normally, insulin activates phosphoserine phosphatase 1, which dephosphorylates and, hence, activates Glut4 (34). Recently, Reusch *et al.* (17) reported that K^+ depolarization or parathyroid hormone treatments increased $[Ca^{2+}]_i$ in isolated adipocytes and that this appeared to result in an increased phosphorylation of Glut4. This effect was likely due directly to changes in $[Ca^{2+}]_i$ because treatment with nitrendipine, which blocks entry of Ca^{2+} into the cell, maintained normal Glut4 levels of phosphorylation. These effects of increased $[Ca^{2+}]_i$ on phosphorylation of Glut4 appeared to be mediated by Ca^{2+}-induced phosphorylation and activation of inhibitor 1, which functions to inhibit phosphoserine phosphatase 1 activity (18). Overall, based on these findings, we predict that the hyperinsulinemia/insulin resistance in the yellow obese mutants causes increased $[Ca^{2+}]_i$ in soleus muscle, which induces increased activity of inhibitor 1. This effect leads to an increase in phosphorylation of PP1, which, in turn, results in an increased phosphorylation and inactivation of the insulin-responsive substrates within the cell, including Glut4. Experiments to directly evaluate the activity of Glut4 and the other insulin-responsive components in the cell will help to confirm this prediction.

While the ectopic expression of agouti causes changes in $[Ca^{2+}]$, it remains unclear how specifically agouti is signaling the responses in soleus muscle described in this report. Within the hair follicle, agouti acts as an antagonist for the binding of α-MSH to its receptor on the melanocyte (8). In this manner, it prevents the α-MSH-induced increases in the level of cAMP within the cell (8) and, hence, elicits its biological action. Since the α-MSH receptor does not appear to be expressed in skeletal muscle (35), agouti cannot be acting in exactly the same manner in muscle as it is in the hair follicle. Another member of the melanocortin receptor family, MCR5, is expressed on skeletal muscle (36). Therefore, it is possible that agouti is functioning on the MCR5 receptor in soleus muscle in a manner similar to that which occurs with the α-MSH receptor in the hair follicle. Alternatively, agouti may act through an interaction with another as-yet-unidentified receptor or possibly by upregulating voltage- and/or receptor-operated Ca^{2+} channels within the cell. Experiments utilizing purified recombinant agouti protein should help to address this issue.

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