Physiol Genomics 20: 173–182, 2005.
First published November 2, 2004; doi:10.1152/physiolgenomics.00145.2004.

ACE gene titration in mice uncovers a new mechanism for ACE on the control of body weight

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Submitted 24 June 2004; accepted in final form 26 October 2004

Heimann, A. S., M. H. Favarato, F. C. Gozzo, V. Rioli, F. R. Carreño, M. N. Eberlin, E. S. Ferro, J. H. Krege, and J. E. Krieger. ACE gene titration in mice uncovers a new mechanism for ACE on the control of body weight. *Physiol Genomics* 20: 173–182, 2005. First published November 2, 2004; doi:10.1152/physiolgenomics.00145.2004.—Mice harboring 1, 2, or 3 copies of the angiotensin-converting enzyme (ACE) gene were used to evaluate the quantitative role of the ACE locus on obesity. Three-copy mice fed with a high-fat diet had lower body weight and peri-epididymal adipose tissue than did 1- and 2-copy mice ($P < 0.05$). On regular diet, 3-copy mice had to eat more to maintain the same body weight; on a high-fat diet, they ate the same but weighed less than 1- and 2-copy mice ($P < 0.05$), indicating a higher metabolic rate in 3-copy mice that was not affected by ANG II AT_1 blocker treatment. A catalytically inactive form of thimet oligopeptidase (EC 3.4.24.15; EP24.15) was used to isolate ACE substrates from adipose tissue. Liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) identified 162 peptide peaks; 16 peptides were present in both groups (1- and 3-copy mice fed with a high-fat diet), whereas 58 of the 72 unique peptides were found only in the 3-copy mice. Peptide size distribution was shifted to lower molecular weight in 3-copy mice. Two of the identified peptides, LVVYPWTQRY and VVYPWTQRY, which are ACE substrates, inhibited in vitro protein kinase C phosphorylation in a concentration-dependent manner. In addition, neurolysin (EC 3.4.24.16; EP24.16) activity was lower in fat tissue from 3- vs. 1-copy mice ($P < 0.05$). Taken together, these results provide evidence that ACE is associated with body weight and peri-epididymal fat accumulation. This response may involve the generation of oligopeptides that inhibit the activity of EP24.16 and other oligopeptidases within the adipose tissue.

insulin resistance; peptides; oligopeptides; EP24.16; angiotensin converting enzyme

CLUSTERING OF CARDIOVASCULAR risk factors including hypertension, diabetes, dyslipidemia, and obesity has long been recognized. Epidemiological data support the association of insulin resistance with these cardiovascular derangements, especially the metabolic syndrome (48), which is consistent with intracellular insulin signaling impairment in these conditions (51).

Major advances have now been made in identifying the components of the homeostatic system that regulate body weight, including several of the genes responsible for animal and human obesity (24). The regulation of food intake and energy expenditure is controlled by a complex neuroendocrine system with important signaling molecules (peptides) including leptin, insulin, resistin, and ghrelin (5, 27, 30). Peptide processing enzymes may also play a role in this chain as illustrated by the development of obesity in mice harboring a defect in the carboxypeptidase E (CPE) gene product, an enzyme required for the processing of numerous neuroendocrine peptides (23). Furthermore, angiotensin-converting enzyme (ACE, EC 3.4.15.1), a zinc metallopeptidase that participates in the activation of the renin-angiotensin system (RAS) and the breakdown of active peptides from the kinin system, has also been associated with the pathogenesis of obesity, insulin resistance, and other related cardiovascular diseases (6, 34, 55). In contrast, the role of other well-known peptide-degrading enzymes, such as thimet oligopeptidase (EC 3.4.24.15; EP24.15) and neurolysin (EC 3.4.24.16; EP24.16), on the regulation of adipose tissue metabolism remains poorly understood (6, 31, 32, 61).

The components of the RAS have been detected in a variety of tissues including kidney, brain, heart, blood vessels, and adipose tissue (3, 6, 20, 35) and may influence pathological states by altering gene expression, cell growth, fibrosis synthesis, and possibly the degree of inflammatory response (9, 37, 38). The adipose tissue contains a fully functional RAS, and ANG II appears to play a role in adipocyte growth and differentiation (21). Activation of the RAS has been shown to be involved with body fat homeostasis (18, 45, 57, 62), and the use of ACE inhibitors improves insulin sensitivity (60).

However, the quantitative role of a single genetic factor influencing a complex phenotype such as obesity is most likely discrete and difficult to identify. The generation of genetically engineered mice harboring 1, 2, or 3 copies of the ACE gene overcomes some of these limitations enabling, under controlled genetic and environmental conditions, testing of the role of the ACE locus on obesity. The results of the present study suggest that the ACE locus influences body weight and peri-epididymal fat accumulation. In addition, we provide evidence that ACE and other peptidases may control body weight via a complex set of novel peptides, some of them identified in the present report.

METHODS

Animals. To investigate the role of ACE in the control of body weight, 3-wk-old male genetically engineered mice carrying either an inactivation or a duplication of the *ACE* locus on chromosome 11 were used. Mice carrying either one (1-copy), two (2-copy), or three (3-copy) functional *ACE* gene copies were studied (33). These mice

Article published online before print. See web site for date of publication (http://physiolgenomics.physiology.org).

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differ as to the ACE locus and represent a controlled genetic model where the role of this locus can be quantitatively assessed under different environmental conditions, e.g., a high-fat diet. The knock-out and knock-in mice lines were maintained by backcross breeding to C57BL/6J mice. Identification of genetically modified offspring were made at 21 days of age by PCR amplification of DNA isolated from ear biopsies as described previously (33). Three-week-old mice were randomized in three groups according to the genotype, and they were either fed with a control diet (5% coconut oil) or a high-fat diet (35.5% coconut oil; Bioserve) for the following 27 wk. Additionally, a group of 3-copy mice fed with a high-fat diet was treated with the AT_1 blocker (losartan, 120 mg/l in the drinking water) for 22 wk (15). All groups had free access to tap water. Throughout the 27 wk, several phenotypes associated with obesity and body weight control were determined.

Weekly glucose levels were determined over 23 wk (from 7 to 30 wk of age). Blood for glucose determinations was collected from the tail vein after 6 h of fasting, always at the same time of the day, using a blood glucose monitor and test strips (Advantage; Roche).

At the end of the experiment, plasma insulin levels were determined (radioimmunoassay using rat insulin antibody; Linco Research) in 40 μl of plasma collected by tail incision in conscious mice after 6 h of fasting. The samples were stored at −80°C for less than 6 mo.

Body weight was determined weekly from 7 to 30 wk of age always in the morning. In a subgroup of animals, 24-h food consumption was determined weekly throughout the study in mice with the same genotype, kept three to a cage.

At 30 wk of age, tissues were harvested while the animals were anesthetized. After perfusing with sterile normal saline, until clear perfusate came out from the right atrium, the left kidney and the peri-epididymal adipose tissue were collected for determination of weight, ACE activity, and for peptide panel analysis.

Care of mice followed institutional guidelines, and the ethics committee of the University of São Paulo Medical School approved the protocol.

Enzyme activity assay. The oligopeptidase activity related to EP24.15 or EP24.16, or both, was determined in duplicate in a continuous assay using the quenched fluorescent substrate [QFS; 7-methoxycoumarin-4-acetyl-Pro-Leu-Gly-Pro-dLys-(2,4-dinitrophenyl)], as previously described (45, 59). ACE enzymatic activity was measured similarly using the internally quenched fluorescent peptide ortho-aminobenzoyl-Phe-Arg-Lys(2,4-dinitrophenol)-Pro-OH (2). The enzymatic activities were normalized by protein concentration determined according to the Bradford (8) assay using bovine serum albumin (BSA) as standard.

Protein kinase C activity assay. The protein kinase C (PKC) activity was determined using a standard procedure PepTag assay for nonradioactive detection of PKC (Promega). It used 0.08 μM of the fluorescent peptide (PLSRTLSVAAK), 15 ng of PKC, and 0.08–8 μM of the LVVYPWTQRY and VVYPWTQRY peptides identified in the adipose tissue. Peptide EFEVLDK without a putative PKC phosphorylation site was used as a control peptide.

Peptide panel. To produce the adipose tissue peptide panel, we used the method described by Rioli et al. (49). Briefly, enzyme (1–5 nmol)-peptide complexes were produced by incubating the peptide extract (16) from the adipose tissue with catalytically inactive EP24.15 in 200 μl of buffer (25 mM Tris·HCl, pH 7.5, containing 125 mM NaCl and 0.1% BSA) for 30 min at room temperature. At the end of this period, the reaction mixture was layered onto a dried Sephadex G-25 column and centrifuged at 1,000 *g* for 2 min. The flow-through solution (≈200 μl) was collected, and the peptide content was analyzed. A control experiment was done performing the assay in the absence of EP24.15.

Peptide sequencing by LC-MS/MS. Liquid chromatography tandem mass spectrometry (LC-MS/MS) analyses were performed in a Q-Tof apparatus (Micromass, Manchester, UK) coupled to a capillary liquid chromatography system (CapLC from Waters). The peptide mixture was desalted online using a Waters Opti-Pak C18 trap column. The mixture of trapped peptides was then separated by elution with a water/acetonitrile 0.1% formic acid gradient through a 75-μm ID capillary column home-packed with C18 silica. Data were acquired in data-dependent mode (DDA), and multiple-charged protonated peptide ions generated by electrospray ionization (ESI) were automatically mass selected and dissociated in MS/MS experiment by 10 up to 30 eV collisions with argon. The product ion tandem mass spectra were processed using MaxEnt3. These data were used to determine whether a peptide is unique. Peptides were considered unique when no other peptide is present that has the same total *m/z* ration and/or *m/z* dissociation values. After these first analyses, peptide sequence was deduced manually with the help of the PepSeq Program (Micromass; Waters, Toronto, Ontario). Typical LC and ESI conditions were flow of 200 nl/min, nanoflow capillary voltage of 3 kV, block temperature of 80°C, and cone voltage of 40 V.

Peptide sequence homology analyses in the database. To identify the putative protein precursors for the sequenced peptides by LC-MS/MS, a protein database (**http://www.ncbi.nlm.nih.gov/blast**) was searched for short nearly exact matches ("Rodentia [ORGN]" format selection), as previously described (1). When the perfect-matching large-protein sequence could not be found for a given peptide, more than one protein precursor was listed, containing part of the peptide identified. To analyze binding sites and consensus motifs, we used the PROSITE algorithm (22).

Statistical analysis. Data are presented as means ± standard error of the mean (SE). Areas under the curves were calculated for each mouse and normalized by the time of the experiment. The averages of the normalized areas are the values of weight and food consumption shown in Fig. 2 and the values of blood glucose shown in Table 1. Two-way ANOVA was used to compare mean values from all groups measured at different times, and one-way ANOVA was used to compare mean values of all groups in one moment only. All statistical analyses were performed using GraphPad Prism Software.

RESULTS

Mice harboring 1, 2, or 3 copies of the ACE gene were fed either a control diet or a high-fat diet for a 27-wk period, from the weaning day until they were 30 wk of age. The weight of the animals given the control diet for this long period was not

Table 1. *Relationship between ACE genotype and obesity phenotypes in mice*

	Control Diet				High-Fat Diet			
	1 copy	2 copies	3 copies	*P* value	1 copy	2 copies	3 copies	*P* value
Body weight	22.4±0.7 (*n*=15)	24.0±0.6 (*n* = 15)	23.2±0.6 (*n* = 10)	0.23	20.6±0.6 (*n* = 11)	19.6±0.8 (*n* = 14)	17.4±0.4 (*n* = 7)	0.0337
PAT weight	0.28±0.06 (*n* = 5)	0.4±0.1 (*n* = 5)	0.32±0.06 (*n* = 2)	0.106	0.39±0.1 (*n* = 6)	0.32±0.07 (*n* = 6)	0.27±0.07 (*n* = 5)	0.0304
Blood glucose	57.7±14.5 (*n* = 6)	64.3±9.5 (*n* = 7)	53.9±5.1 (*n* = 5)	0.260	73.2±3.4* (*n* = 8)	72.8±6.9* (*n*=6)	76.2±4.6* (*n* = 5)	0.492
Blood insulin	0.45±0.2 (*n* = 6)	0.39±0.19 (*n* = 4)	0.27±0.06 (*n* = 5)	0.2119	1.1±0.05* (*n* = 8)	0.75±0.5* (*n* = 6)	0.69±0.08* (*n* = 5)	0.06

Values are means ± SE. Please note that the asterisks correspond to significant differences in control vs. high-fat diet (*$P<0.05$), whereas the *P* value in the last column corresponds to the genotype effect within each regimen. PAT, peri-epididymal adipose tissue; ACE, angiotensin-converting enzyme.

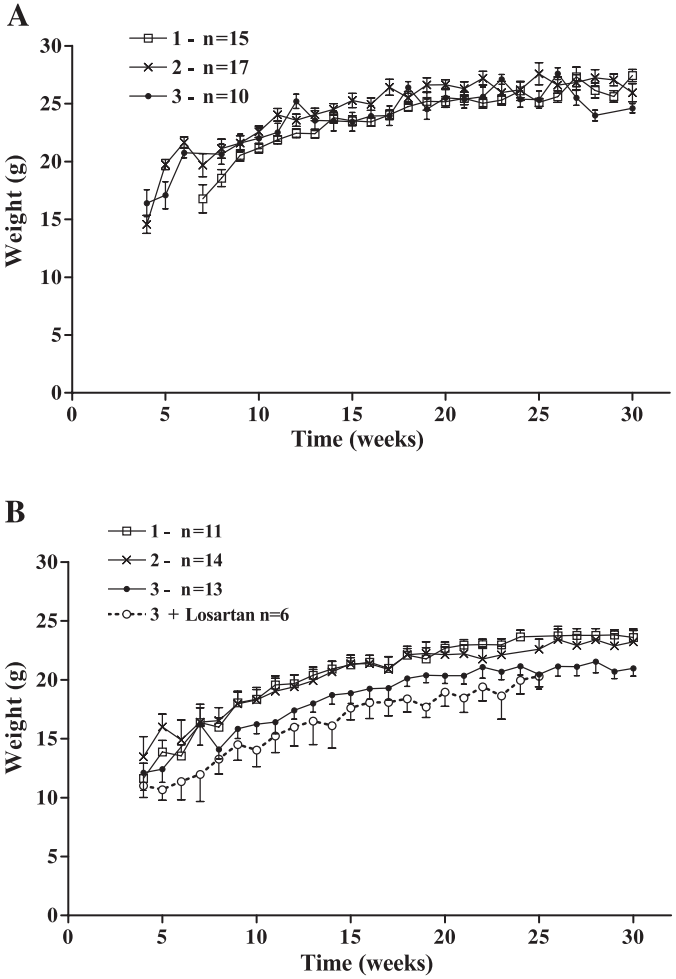

Fig. 1. Effect of diet on weekly body weight measurements of mice harboring 1, 2, or 3 copies of the ACE gene over a 27-wk period. *A*: results from mice fed a control diet. *B*: results from mice fed a high-fat diet. Note that with a control diet, body weight remains the same regardless of the ACE genotype, whereas with a high-fat diet, 3-copy mice have smaller body weight compared with that in the 1- and 2-copy groups. *B*: a second group of 3-copy mice receiving the ANG II AT_1 receptor blocker (losartan, 120 mg/l in the drinking water) was included to test whether this response was mediated by ANG II acting on the AT_1 receptor. Data are presented as mean and SE from weekly determinations. The labels 1, 2, and 3 refer to the number of copies of the ACE gene in each group.

influenced by the ACE genotype (Fig. 1*A*). In contrast, under the high-fat regimen, the 3-copy mice had smaller body weight than both 1- and 2-copy mice (Fig. 2, *A* and *C*, and Table 1). It is important to emphasize that in both, high-fat or control diet, normalizing body weight by food consumption shows that mice with 3 copies of the ACE gene are thinner and that a high-fat diet raises the relative body weight of all mice independently from the genotype (Fig. 2*C*). Body weight was also measured in a subgroup of 4-wk-old mice fed with a high-fat diet with 1, 2, or 3 copies of the ACE gene, and no differences were observed among groups [11.6 ± 1.3 (n = 8), 13.5 ± 0.7 (n = 5), 12.1 ± 1.5 g (n = 5), P = 0.68, respectively]. Areas under the curve were calculated for each mouse and normalized by the time of the experiment. The averages of the normalized areas are the values of weight and food consumption shown in Fig. 2, *A* and *B*. In agreement with this finding, the peri-epididymal adipose tissue weight of 3-copy mice fed a high-fat diet was significantly lower compared with that of 1-copy mice (Table 1). Glucose values, derived from weekly data determined throughout the study, and insulin levels, measured once at the end, were higher in animals fed a high-fat diet compared with that in the control diet group, regardless of the ACE genotype (Table 1). No internal differences were observed among these groups regarding glucose levels (Table 1). However, insulin levels were lower in 3-copy mice fed a high-fat diet compared with 1-copy mice (Table 1). These findings are consistent with the expected changes observed in humans, where a high-fat diet is known to be associated with insulin resistance and obesity (4, 9).

Possible scenarios to explain these differences in body weight include metabolism changes, food consumption, or both. In this context, mice fed the control diet and harboring 3 copies of the ACE gene had higher food consumption throughout the study compared with 1-copy mice (Fig. 2*B*). On the other hand, with a high-fat diet, no differences in food consumption were observed among the groups (Fig. 2*B*). Interestingly, 3-copy mice required a higher food intake to maintain the same body weight compared with 1- and 2-copy mice receiving the control diet, whereas during high-fat feeding, the 3-copy mice had smaller body weight than 1- or 2-copy mice despite the same food intake (Fig. 2). Thus, in both conditions, the data are consistent with the idea that the 3-copy mice have a higher metabolic rate. Moreover, ACE enzymatic activity increases proportionally with the number of copies of the ACE gene in the kidneys and adipose tissues (Fig. 3*A*). Thus a separate group of 3-copy mice was treated for 22 wk with the ANG II AT_1 antagonist (losartan, 120 mg/l in drinking water) while on a high-fat diet to test whether the changes observed with this group were secondary to higher levels of ANG II acting on the AT_1 receptor. Body weight of these mice remained the same while receiving a high-fat diet, regardless of the AT_1 antagonist treatment (Figs. 1 and 2). Altogether, these data suggest that ACE 3-copy mice have a higher metabolic rate compared with 1- and 2-copy groups and that AT_1 receptor-mediated events appear not to be involved.

To address possible mechanisms whereby increased ACE may lead to higher metabolism and decreased fat weight in 3-copy vs. 1-copy mice, we used the approach based on a catalytically inactive form of EP24.15 previously described by us (49) to search for putative peptide content dissimilarities in the adipose tissue from mice with 1 or 3 copies of the ACE gene (Fig. 4). Using this method where the catalytically inactive enzyme behaves like bait, we identified 162 peptide peaks LC-ESI-MS/MS considering their mass/charge (m/z) ratio, and only 16 peptides were identical and present in both groups. In the 1-copy group, 72 unique peptides were identified, whereas 58 unique peptides were found in the 3-copy mice (Fig. 5*A*). The complete amino acid sequence of several of these peptides suggests that most of the peptides originated from intracellular precursor proteins (Table 2). Although the peptides LVVYPWTQRY and VVYPWTQRY have already been described (25, 29, 49), the vast majority of the peptides we sequenced are novel (Table 2). Peptide size distribution indicates that 3-copy mice fed a high-fat diet give rise to lower molecular weight peptides compared with 1-copy mice (Fig. 5*B*). This mass shift suggests that 3-copy mice are producing more of lower molecular weight peptides. Because short peptides (oligopeptides)

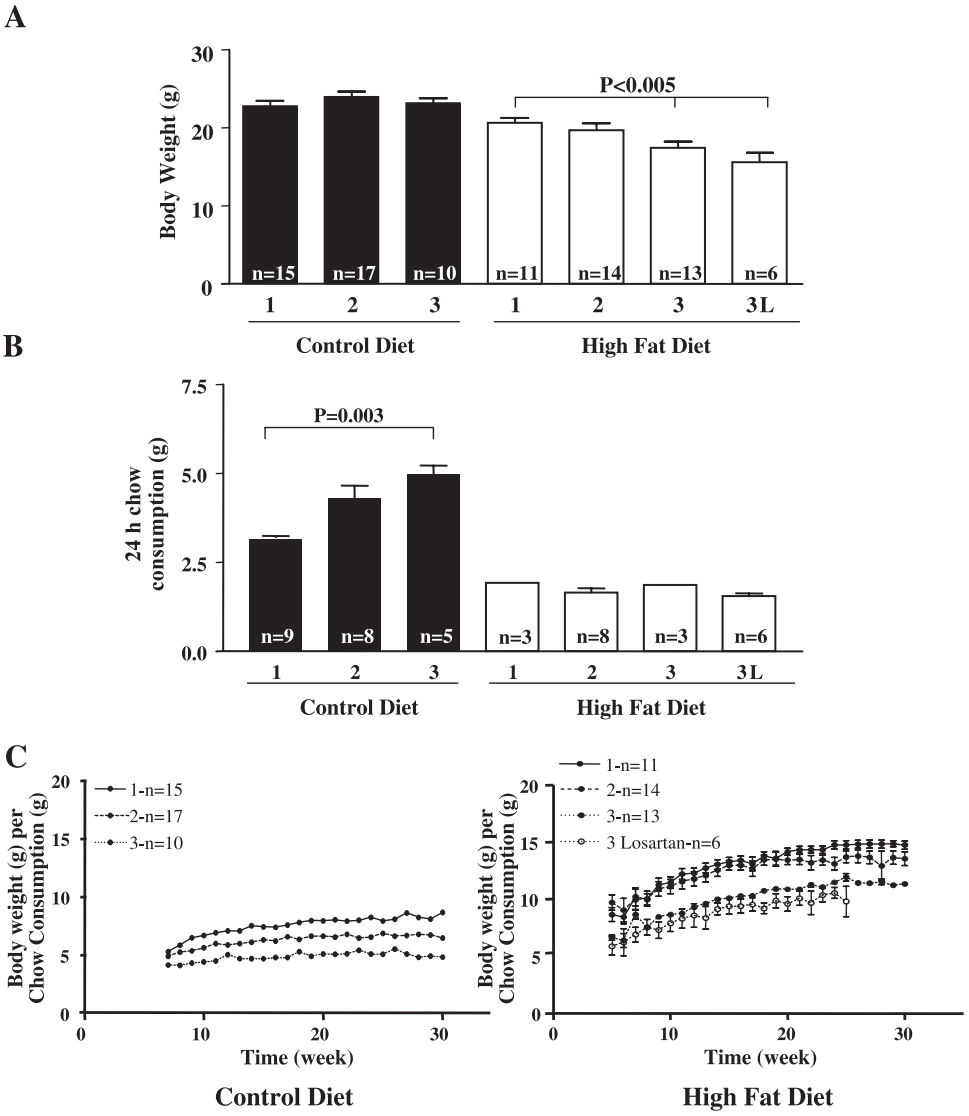

Fig. 2. Body weight and food consumption in mice harboring 1, 2, or 3 copies of the ACE gene. *A*: body weight of mice fed a control or high-fat diet. Note that mice fed a control diet have the same body weight, but mice harboring 3 copies of the ACE gene fed a high-fat diet have lower body weight than 1- and 2-copy mice. The response to ANG II AT_1 receptor blocker (L, losartan) in the presence of a high-fat diet is also shown. *B*: food consumption of mice fed with a control or high-fat diet. Note that 3-copy mice fed a control diet have higher food consumption, but mice fed a high-fat diet have the same food consumption independent of the ACE genotype or the ANG II AT_1 receptor blocker administration. *C*: normalized body weight by food consumption. Note that by normalizing the body weight by the food consumption it becomes clear that mice with 3 copies of the ACE gene are thinner and that a high-fat diet raises the relative body weight of all mice independently from the genotype. The columns in *A* and *B* represent the mean and SE of the body weight and food consumption determined weekly throughout the study.

have been described both as substrates and inhibitors of the intracellular oligopeptidase (47) while dipeptides have been shown to efficiently inhibit the EP24.16 (19), it is conceivable that dipeptides inhibiting EP24.16 or other oligopeptidases in the adipose tissue may affect fat metabolism. Indeed, the adipose tissue activity of EP24.16 was lower in the 3-copy mice compared with that in the 1-copy mice (Fig. 3*B*). Moreover, peptides LVVYPWTQRY and VVYPWTQRY are ACE substrates (49) and have putative PKC phosphorylation sites (underlined; PROSITE). We then tested whether these two peptides could inhibit in vitro phosphorylation of a synthetic standard PKC substrate in a concentration-dependent manner (Fig. 6). The data show that both peptides compete in vitro for targets of PKC and that LVVYPWTQRY is more efficient than the VVYPWTQRY at inhibiting PKC phosphorylation of a synthetic standard peptide.

DISCUSSION

The major finding of the present study is that ACE 3-copy mice have lower body weight compared with 1- or 2-copy mice with chronic high-fat intake, indicating that ACE is involved in body weight control in mice. As a result of ACE activation, a large number of distinctive novel peptides have been identified in adipose tissue and suggested to affect intracellular oligopeptidase activity. Therefore, these findings are consistent with the idea that the ACE gene may contribute to the regulation of body weight through a complex set of peptides produced within the adipose tissue.

The involvement of RAS dysfunction as an underlying factor in the genesis or maintenance of cardiovascular diseases is a very appealing concept, and it is reinforced by the success of different classes of drugs whose main targets are components of the RAS (34). Therefore, it is reasonable to assume that increased ACE gene expression may confer augmented susceptibility on cardiovascular events or influence important homeostatic control systems like body weight control mechanisms. In humans, plasma ACE activity is associated with the common ACE I/D polymorphism [individuals homozygous for the I allele have lower plasma ACE activity than individuals homozygous for the D allele (13)] and has been instrumental in testing the hypothesis that the ACE locus is associated with a cardiovascular condition such as obesity. Results, however,

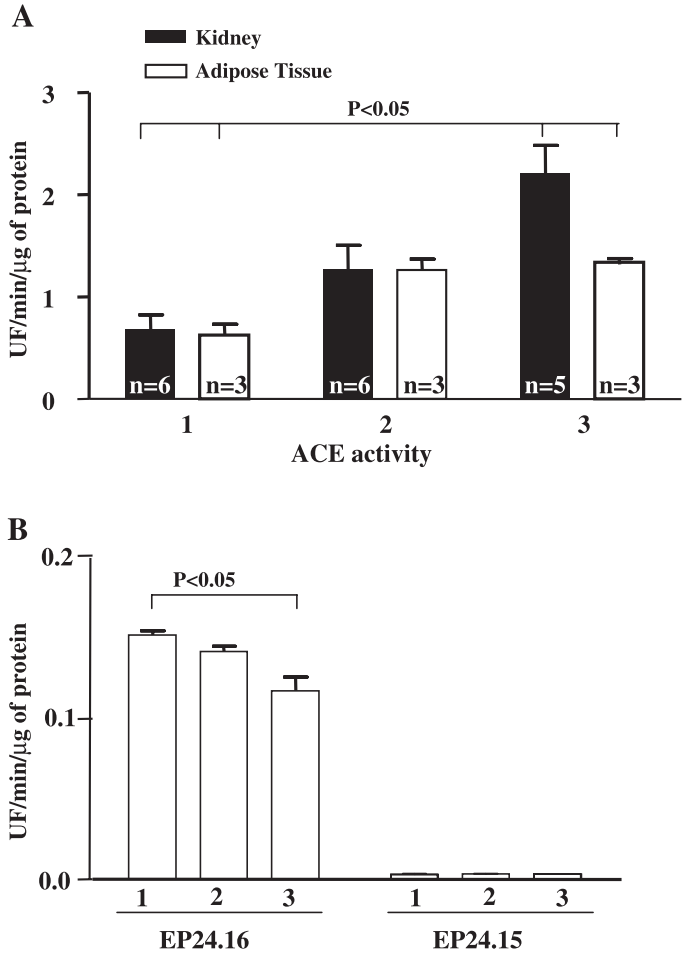

Fig. 3. Enzymatic activity. *A*: ACE enzymatic activity in the kidney and adipose tissue of mice fed a high-fat diet. *B*: thimet oligopeptidase (EC 3.4.24.15; EP24.15) and neurolysin (EC 3.4.24.16; EP24.16) enzymatic activity in the adipose tissue of mice fed a high-fat diet. Note that 3-copy mice had higher ACE and lower EP24.16 activity, and no EP24.15 activity occurred in the adipose tissue.

have been contradictory, and while the D allele has been associated with overweight and abdominal adiposity in some studies (13, 54), others have failed to reproduce such an association (50, 57). The genesis of obesity is associated with a complex interplay between environmental and genetic factors whose individual contribution must be discrete. In this context, the generation of mice harboring 1, 2, or 3 copies of the ACE gene enables one to test the quantitative role of ACE on this phenotype under controlled genetic and environmental conditions.

We verified that fed a chronic high-fat diet, 3-copy mice have similar food consumption but lower body weight compared with that of 1- or 2-copy mice. In contrast, with a regular diet, 3-copy mice show larger food consumption and similar body weight compared with that of 1- or 2-copy mice (Fig. 2). Together, these results indicate that mice harboring 3 copies of the ACE gene have higher energetic metabolism associated with higher tissue ACE activity. However, this high-fat regimen did not lead to an overt increase in body weight. This has already been described and may be a consequence of the particular genetic background of these mice. These animals have the C57BL/6J background, which is a recombinant inbred strain derived from crosses of C57BL/6ByJ and BALB/cByJ (39). It is now well established that inbred mouse strains differ in their susceptibility to high-fat/high-cholesterol diet-induced obesity and atherosclerosis (42–44), with C57BL/6 mice showing susceptibility and strains A/J, BALB/c, and C3H/He showing resistance to these disorders. The fact that C57BL/6J is derived from crosses of C57BL/6ByJ (susceptible to high-fat diet-induced obesity) and BALB/cByJ (resistant) gives rise to a heterogeneous strain regarding metabolic adaptation. Burcelin et al. (11) have reported that C57BL/6J mice fed a high-fat diet for 9 mo display heterogeneous metabolic adaptation. Three well-defined phenotypes can be distinguished on the C57BL/6J fed with a high-fat diet: obese, normal weight, and lean. Importantly, whatever is their phenotype, all of the high-fat diet mice became insulin resistant, also observed in the present study (Table 1). Another explanation for the lack of overt obesity may be related to the fact that a high-fat diet is less palatable to these mice. Indeed, evidence exists that BALB/cByJ mice lose their appetite on a high-fat diet, and C57BL/6J mice can have a decreased appetite after 18 days on the diet, but, interestingly, this behavior is not observed in the C57BL/6ByJ mice (53).

A high-fat diet can be considered a model of metabolic syndrome (27, 30), as it increases insulin and glucose levels (Table 1). Insulin sensitivity is a well-known factor influencing the control of body weight (30), and the lower blood glucose and insulin levels in the 3-copy mice suggest that these mice have higher insulin sensitivity compared with 1- or 2-copy mice, especially with a high-fat diet (Table 1). We do not know whether changes in insulin sensitivity are a primary or secondary event, but under the present controlled genetic and environmental conditions the main experimental difference resides within the ACE locus highlighting the importance of ACE for this response. These findings are consistent with the correlation of the D allele with higher insulin sensitivity in patients with insulin resistance syndrome (45).

Increasing or decreasing the number of copies of the ACE gene in mice is associated with proportional changes in ACE activity and ANG II levels (14). Under normal conditions, these changes do not cause gross abnormalities as illustrated by maintenance of blood pressure levels in these animals (33, 58). In contrast, we have demonstrated that in response to a specific noxious stimulus, such as a chronic high-fat diet, a significant effect on body weight control occurs, which is a phenotype usually under well-maintained homeostatic equilibrium. It is unlikely that this is a particular response of this animal model, but direct validation of these findings in humans remains a challenge. Several scenarios can be considered to explain these findings. It is possible that higher ACE activity can lead to increased ANG II levels in the central nervous system (CNS) or in the adipose tissue, or in both of these. Some studies in rats that chronically received ANG II systemically or injected in the CNS (47) have shown that ANG II acting via AT_1 receptors can increase metabolic rate and decrease body weight, which can lead to higher insulin sensitivity. However, chronic treatment of 3-copy mice with the ANG II AT_1 receptor antagonist resulted in no change in body weight compared with that in the untreated animals, providing no evidence for the influence of ANG II AT_1-mediated effects on this response. In contrast, one cannot exclude the possibility that these findings are due to

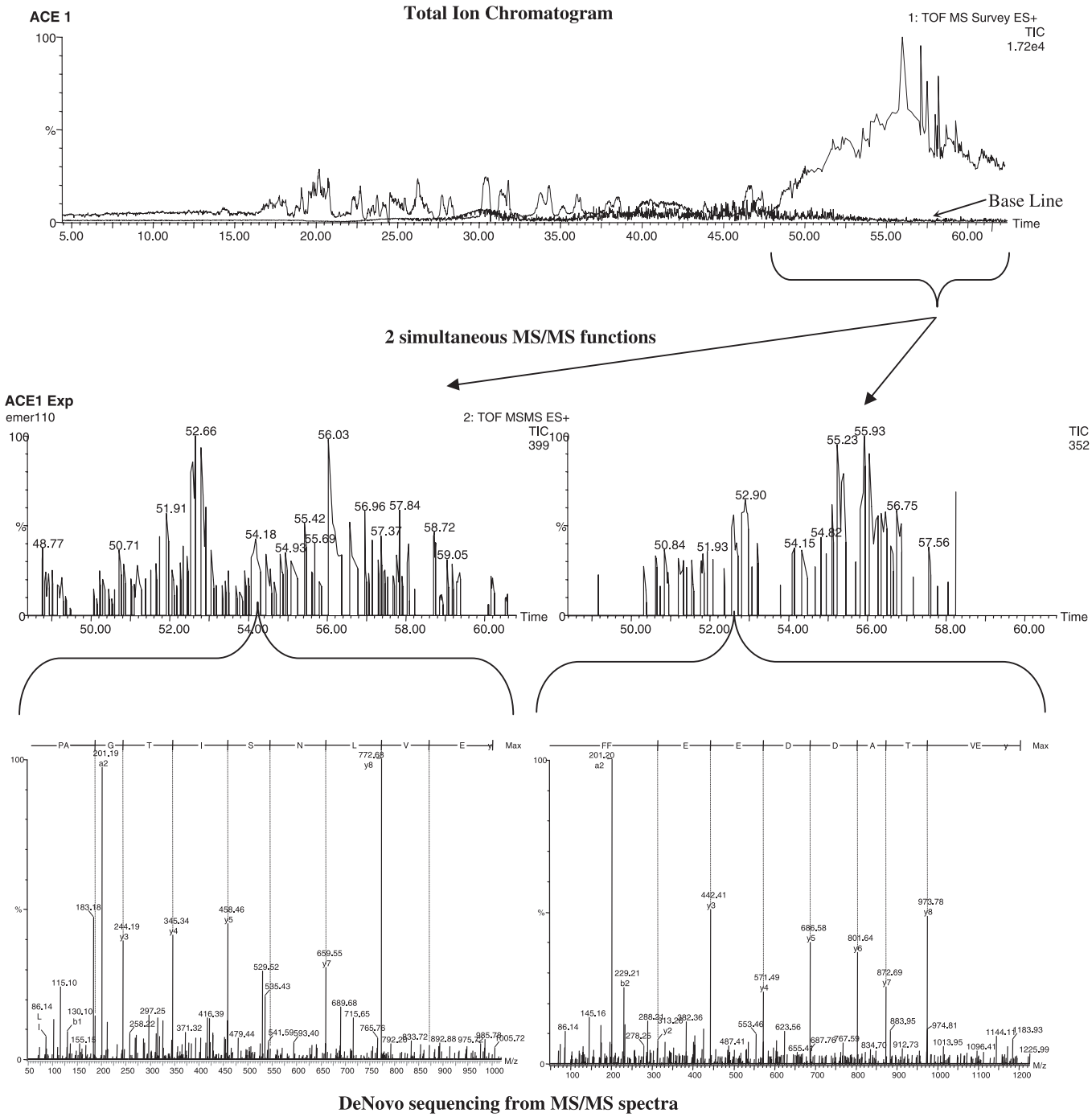

Fig. 4. Liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) chromatogram showing the mice peptides arrested by inactive EP24.15. Following incubation of the mice adipose tissue peptide extract with inactive EP24.15, the enzyme-peptide complexes were eluted through a Sephadex G25 column and analyzed by ESI-MS/MS, as detailed in the METHODS. Control experiments were done in the absence of inactive peptidases.

ANG II AT_2-mediated effect or secondary to a decrease in kinin levels associated with an increased degradation of the active peptides.

Other peptide hormones besides ANG II affect body weight regulation and could be influenced by ACE. Ghrelin is a peptide released mainly from the stomach and acts in the hypothalamus to stimulate food intake (5). Leptin and insulin also act centrally in the hypothalamus to inhibit food intake, increase metabolic rate, and reduce body weight (5). While insulin, leptin, and ghrelin trigger the signaling for food consumption, other neuropeptides including neuropeptide Y, agouti protein, α-MSH, CART, hypocretin 1 and 2, interleukin-1β, and melanocortin (MC) are involved in other downstream events related to body weight regulation (5). Distinctive peptidases/proteases responsible for regulating peptide hormone levels have also been involved in body weight control and obesity phenotype (6, 23, 27). As an example, dipeptidyl peptidase IV (DP-IV), a member of the prolyl oligopeptidase

A

	Total peptides	Identical peptides	Total distinguished peptides
ACE 1	88	16	72
ACE 3	74	16	58

B

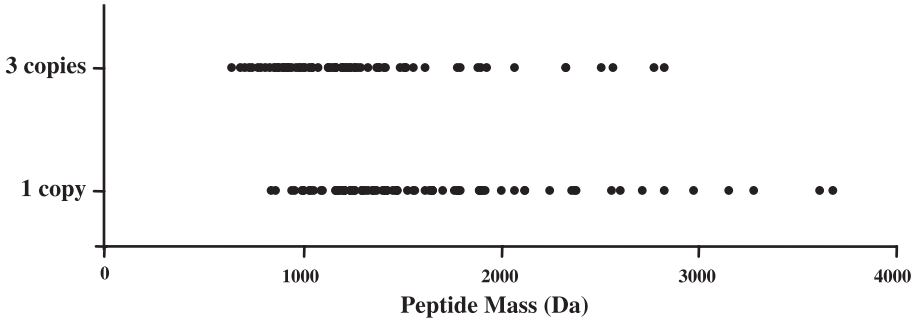

Fig. 5. Peptide identification by LC-ESI-MS/MS. Following incubation of the mice adipose tissue peptide extract with inactive EP24.15, the enzyme-peptide complexes were eluted through a Sephadex G25 column, separated by CapLC chromatography and sequenced by ESI-MS/MS. *A*: the number of peptide peaks detected in the adipose tissue of mice with 1 or 3 copies of the ACE gene. *B*: the relationship between mass distribution of the peptides and in mice harboring 1 or 3 copies of the ACE gene.

family of peptidases, is involved in the metabolic inactivation of a glucose-dependent insulinotropic hormone, glucagon-like peptide 1 (GLP-1), and other incretin hormones. Therefore, mice lacking the gene encoding DP-IV are protected against the development of obesity and insulin resistance, which suggests that DP-IV has a significant impact on body weight control and energy homeostasis (17).

We have recently demonstrated the use of catalytically inactive oligopeptidases, neurolysin (EP24.16) and endopeptidase 24.15 (EP24.15), which can be used to isolate novel ACE substrates from rat brain homogenates (49). This similar substrate specificity among ACE, EP24.15, and EP24.16 is probably related to their structural similarity among the catalytic cleft (10, 40). As a functional consequence, we hypothesized that oligopeptidases (EP24.15 and/or EP24.16) and ACE act in concert to regulate peptide levels within adipose tissue. Therefore, using the inactive form of the EP24.15, we have now isolated a series of peptides from the adipose tissue extracted from mice fed a high-fat diet containing 1 or 3 copies of the ACE gene. ACE is a membrane-bound dipeptidyl-carboxypeptidase, and its long-term increased activity should produce a higher amount of shorter peptides that are products of the enzymatic reaction and a lower amount of longer peptides that are substrates of the enzyme. Indeed, adipose tissue isolated from mice with 3 copies of the ACE gene contains smaller peptides compared with that in 1-copy mice (Fig. 5*B*). Moreover, increased ACE activity should release more dipeptides due to its sequential action over the COOH-terminal of peptide substrates. Such dipeptides can be transported into the cells by H^+-coupled energy-dependent transporters, which is a mechanism that plays a role in the oral absorption of ACE and renin inhibitors (36). Interestingly, several dipeptides are known to inhibit the catalytic action of peptidases, including the EP24.16 (19), DP-II, and DP-IV. The latter is already known to be involved in obesity and insulin resistance phenotypes (17). In fact, 3-copy mice have lower EP24.16 activity in their adipose tissue (Fig. 3*B*) compared with that in 1- or 2-copy mice. These data support the hypothesis that dipeptides generated by ACE systemically or in the adipose tissue can inhibit EP24.16, which is mainly localized within cells (45, 46). Also, it has been described that an increase in ACE activity reduces EP24.11 activity (41). Inhibition of intracellular EP24.15 activity has been previously demonstrated to affect antigen presentation through MHC class I (52). Therefore, it seems plau-

Table 2. *Analyses of the sequenced peptides by homology with proteins in the database*

	PROSITE	Peptide Sequenced	Putative Precursor Protein	Biological Activity
1 Copy	No hits found	**EVLNSITGAP**	Expressed sequence AW049900 [*Mus musculus*]	unknown
	PKC_PHOSPHO_SITE	**VVYPWTQRY**	Hemoglobin beta chain	Increased after ischemia (29)
	CK2 PHOSPHO_SITE	**EVTADDEEFF**	9030416H16Rik protein [*Mus musculus*]	unknown
	PKC_PHOSPHO_SITE	**LVVYPWTQRY**	Hemoglobin beta chain	Specific binding to opioid receptor, guinea pig ileum contraction (29)
	No hits found	**HISGEPCP**	Hypothetical protein A830007M12 [*Mus musculus*]	unknown
	GLYCOSYLATION	FNNTAEL	BBP-like protein 1 [*Mus musculus*]	unknown
3 Copies	No hits found	LLEQV	Unnamed protein product [*Mus musculus*]	unknown
	No hits found	KSEAMWH	SH3-domain binding protein 4 [*Mus musculus*]	unknown
	No hits found	QVERVP	L-Lactate dehydrogenase A-like [*Mus musculus*]	unknown
	No hits found	STVNH	RIKEN cDNA 3222402P14 [*Mus musculus*]	unknown

The protein database (**http://www.ncbi.nlm.nih.gov/blast**) was searched for short, nearly exact matches on Rodentia organisms; The PROSITE algorithm was used to search for motifs. Full sequences are in bold font. Phosphorylation sites are underlined.

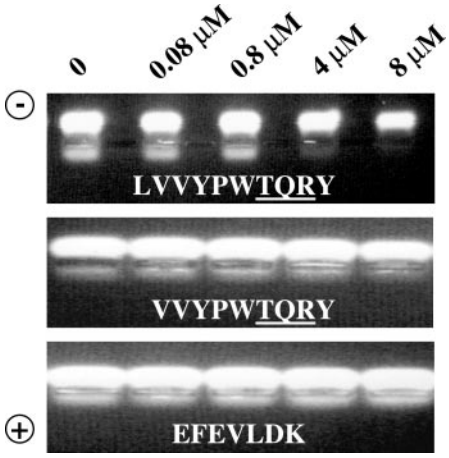

Fig. 6. Competition of peptides identified in the adipose tissue by PKC standard substrate. PepTag C1 peptide (0.08 μM) was incubated as described in the standard PKC assay (Promega) with 15 ng of PKC in a final volume of 25 μl for 30 min at room temperature. The reaction was stopped by heating to 95°C for 10 min. Phosphorylated (+) and nonphosphorylated (−) standard peptides were then separated on a 0.8% agarose gel at 100 V for 15 min. The effects of peptides LVVYPWTQRY and VVYPWTQRY (putative phosphorylation site is underlined) and EFEVLDK (without a putative phosphorylation site) were analyzed at a concentration ranging from 0.08–8 μM. The phosphorylated standard peptide migrated toward the anode (+), while nonphosphorylated peptide migrated toward the cathode (−). Note a proportional reduction on standard peptide phosphorylation by PKC in the presence of increased concentrations of LVVYPWTQRY and VVYPWTQRY, whereas EFEVLDK shows no such effects. The agarose gel was directly photographed on a UV light transilluminator.

sible that EP24.16 inhibition is interfering with normal intracellular peptide metabolism within the adipocyte, which ultimately may lead to metabolism rate augmentation.

A growing body of evidence suggests that peptides may play an important role in intracellular processes in addition to their well-known receptor-mediated functions. For example, calmodulin-dependent protein kinase II (CaMKII), a second-messenger-responsive multifunctional protein kinase that is important for the control of a variety of cellular functions in the CNS, can be inhibited by a 13 amino acid peptide (KKALRRQEAVDAL), designated as autocamtide-2-related inhibitory peptide (AIP) (28). The c-Jun NH_2-terminal kinase, a member of the stress-activated group of mitogen-activated protein kinases (MAPKs), is inhibited by the addition of a bioactive cell-permeant peptide, decreasing intracellular c-Jun NH_2-terminal kinase signaling, which confers long-term protection to pancreatic β-cells from IL-1β-induced apoptosis (7). This intracellular function of peptides can be explained based on protein-protein interactions related to cell signaling, which are frequently mediated by short, unstructured sequences, which specifically interact with peptide binding domains (46). Therefore, many signaling pathways do so by altering the phosphorylation state of tyrosine, serine, or threonine residues of target proteins. Recently, it has become apparent that regulatory mechanisms exist to influence where and when protein kinases and phosphatases are activated in the cell. The Ht31 peptide, derived from a human thyroid cAMP-kinase-associated protein (AKAP), has been already used to disrupt PKA localization (12) showing that peptides having binding sites to other proteins can interfere with intracellular signaling. Moreover, it has been shown that peptides containing known export or shuttling domains of candidate adapter proteins (the NES, M9, and HNS sequences) can selectively inhibit mRNA exporting from nucleus to cytoplasm (26), showing that intracellular peptides can ultimately be regulating protein synthesis. Peptides can also modulate enzymatic activity acting as a purely competitive inhibitor (25). Therefore, normal cell function could be affected as a consequence of intracellular peptide imbalance. Among the peptides identified, the ones from the 1-copy ACE mice have putative sites for posttranslational modification (i.e., phosphorylation and ASN glycosylation; Table 2). Peptides LVVYPWTQRY and VVYPWTQRY were both confirmed herein to compete with a synthetic substrate for PKC phosphorylation, an important second messenger on the insulin signaling (Fig. 6). Therefore, within the adipose tissue LVVYPWTQRY and VVYPWTQRY could be competing with regular protein targets for PKC phosphorylation, altering adipocyte fat metabolism in the 1-copy group, the ones with higher insulin resistance (Table 1). Although a large number of peptides need to be sequenced before the ACE phenotype can be directly related to the generation of a distinctive set of peptides with potential to compete with regular protein targets for posttranslational modifications, the present data suggest that chronic (constant) generation of such species of peptides may change the pattern of protein posttranslational modifications (e.g., phosphorylation) within the adipose tissue cells. Insulin receptor substrate 1 (IRS1) is a substrate for protein kinase CK2 (56) with implications in the alterations observed in insulin resistance and consequentially in body weight. The CK2 binding site of some of the peptides identified in the present study could be involved in the lower insulin sensitivity found in the 1-copy mice fed a high-fat diet. Also, we can speculate that other peptides with putative protein kinase phosphorylation sites could be regulating insulin sensitivity and intracellular metabolism in the 3-copy mice.

Taken together, it is tempting to speculate that EP24.16 inhibition induces an increase in the concentration of a complex set of peptides, which could then compete with specific proteins for posttranslational modifications (e.g., phosphorylation) within the adipose tissue, ultimately leading to a decrease in fat accumulation in the 3-copy ACE mice.

Finally, the results shown herein provide evidence that the ACE locus is associated with body weight and peri-epididymal fat accumulation in mice. The proposed mechanism whereby ACE controls body weight may involve the generation of dipeptides that inhibit the activity of EP24.16 and other oligopeptidases within the adipose tissue. Identification and characterization of these peptides may provide opportunities to better understand this complex phenotype.

ACKNOWLEDGMENTS

We thank Dr. Joel Claudio Heimann, Dr. Maria L. M. Barreto-Chaves, and Dr. Alexandre C. Pereira for valuable suggestions and critical reading of the manuscript and Dr. O. Smithies for providing the genetic modified mice.

GRANTS

This work was supported by grants from the São Paulo State Research Foundation (FAPESP) to J. E. Krieger (grants 01/00009-0 and 00/11176-2) and to E. S. Ferro (grants 99/01983-9 and 01/07544-9) and a grant from the Brazilian National Research Council to J. E. Krieger (CNPq 471219/01-0). M. H. Favarato and A. S. Heimann are supported by FAPESP fellowships for undergraduate and graduate studies (grants 03/01062-8 and 01/11478-1, respectively). J. E. Krieger and E. S. Ferro are supported by research fellowship

awards from CNPq. A. S. Heimann is a PhD student in the Physiopathology Program at the University of São Paulo Medical School.

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