Neutral Aminopeptidase and Dipeptidyl Peptidase IV Activities in Plasma of Monosodium Glutamate Obese and Food-deprived Rats

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Biometric parameters, glycemia and activity levels of plasma neutral aminopeptidase (APN) and dipeptidyl peptidase IV (DPPIV) were measured in monosodium glutamate obese and food-deprived rats (MSG-FD), to analyze the involvement of these enzymes in such situations. Plasma APN was distinguished as sensitive (PSA) (K_m = 7.8 × 10^{-5} mol/l) and predominantly insensitive (APM) (K_m = 21.6 × 10^{-5} mol/l) to puromycin, whereas DPPIV was sensitive (DPPIV-DS) (K_m = 0.24 × 10^{-5} mol/l) and predominantly insensitive (DPPIV-DI) (K_m = 7.04 × 10^{-5} mol/l) to diprotin A. Although unchanged in the MSG and food-deprived animals, APM activity levels were closely correlated with body mass, Lee index, and mass of retroperitoneal fat pad in the food deprived, but not in the MSG animals. DPPIV-DI activity levels decreased by 33% and were correlated with body mass, Lee index, and mass of periepididymal fat pad in the food-deprived MSG rats. These data suggest that APM and DPPIV-DI are respectively related to the downregulation of somatostatin in food-deprived rats, and to the recovery of energy balance in MSG obese rats during food deprivation.

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INTRODUCTION

In obesity and food deprivation, the lipolysis and proteolysis rates are altered (1,2). Exopeptidases are the main enzymes capable of releasing free amino acids as the final product of peptide hydrolysis, and the content of free amino acids is one of the limiting factors of proteolysis (3). Furthermore, several peptides are known to exert significant effects on the nutritional status and energy balance, and enzyme hydrolysis by exopeptidases leading to inactivation or processing of these peptides has been assumed to be a limitation for their biological functions (4). Substance P, somatostatin, angiotensin III, vasopressin, kallidin, dinorphin, leu- and met- enkephalin and endorphin are susceptible to hydrolysis by neutral aminopeptidase (APN) (5–7). Glucagon-like peptide types 1 and 2, glucose-dependent insulinotropic polypeptide or gastric inhibitory peptide, peptide YY (1-36), neuropeptide Y (1-36) (NPY 1-36), endorphin-2, dinorphin, substance P, and growth hormone-releasing hormone are susceptible to hydrolysis by dipeptidyl peptidase IV (DPPIV) (8–17). DPPIV (EC 3.4.14.5) is a serine-type protease and APN (EC 3.4.11.2) is a type II metalloprotease. Both are thought to exhibit various isozymes, including soluble forms in serum, with different functions and implications in the pathogenesis of a variety of diseases, and are therefore considered attractive targets for inhibition therapy (6,18–20). To determine the influence of disrupted control of nutritional state and energy balance on APN and DPPIV, their plasma activity levels and body mass, naso-anal length, Lee index, mass of periepididymal and retroperitoneal fat pads, and glycemia values were measured in monosodium glutamate (MSG) obese and food-deprived rats.

METHODS AND PROCEDURES

Animals, treatments and Lee index and blood glucose measurements

As illustrated in **Figure 1**, immediately after birth, male Wistar rats were housed with a lactant female in a polypropylene box (inside length × width × height 56 cm × 35 cm × 19 cm), with food and tap water ad libitum, in a ventilated container (Alesco, Monte Mor, Brazil), with controlled temperature (24 ± 2 °C), relative humidity (65 ± 1%), and 12:12 h light/dark photoperiod (lights on at 6:00 AM). Twenty-four hours after birth, the animals received a daily subcutaneous bolus injection of L-glutamic acid monosodium salt (Sigma, St Louis, MO) in saline 0.9% (4 mg/g body weight) in the cervical region, between 7:30 and 9:00 h of light period, at a maximum volume of 0.2 ml (MSG animals), until they were 10 days old. At 22 days, the animals were weaned and the female was removed from the cage (21). At 90 days, glycemia was measured in

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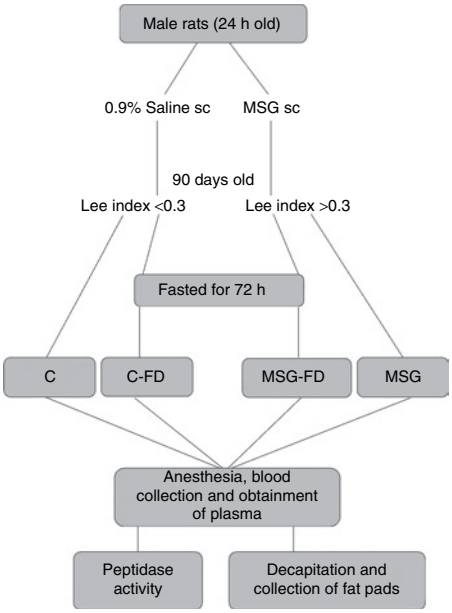

Figure 1 Schematic drawing of experimental procedures. MSG, monosodium glutamate.

the second drop of blood, obtained without pressure from a small cut in the tip of the tail, using an Accu-Chek advantage apparatus (Roche, Nutley, NJ), and the obesity was determined by the Lee index, calculated by body mass $(g)^{0.33}$/naso-anal length (cm) (22,23). MSG obese rats with a Lee index >0.3 were then selected. Rats of the same age and strain, receiving 0.9% saline under the same experimental conditions, with a Lee index ≤0.3, were considered normal (C). Half of both groups were submitted to food deprivation, resulting in MSG-FD (obese animals fasted for 72 h) and C-FD (normal animals fasted for 72 h). Food deprivation was performed by transferring pairs of animals, between 7:30 and 9:00 h of light period, into metabolic cages, where they were housed without food and with water ad libitum for 72 h. After this period, body mass, naso-anal length and glycemia were measured again in MSG-FD and C-FD.

The animal care and handling procedures used were in accordance with the guidelines of the Brazilian College of Animal Experimentation and were approved by the Ethics Committee of the Instituto Butantan (291/06).

Obtaining plasma and collection of fat pads

After these procedures, all the rats were anesthetized with a solution containing ketamine chlorhydrate (König, Avellaneda, Argentina) (100 mg/ml) and xylazine chlorhydrate (Vetbrands, Jacareí, Brazil) (100 mg/ml) by intraperitoneal injection (0.2 ml/100 g of body mass) between 4:00 and 6:30 h during the light phase. Next, 3 ml of individual blood samples was immediately obtained from the left ventricle of each rat, using a heparinized syringe. The individual blood samples were immediately centrifuged at 3,000 *g*, 4 °C for 20 min (H-240 microcentrifuge; Hsiangtai Machinery, Taipei City, Taiwan) to obtain plasma, which was stored at −80 °C for a maximum period of 2 days, until its use for measuring enzyme activity and protein content. The animals were then killed by decapitation. Whole fatty deposits in the retroperitoneal and periepididymal regions were quickly removed by laparotomy, and their wet weights determined.

Protein and peptidase activity

Protein content was measured at 630 nm, in triplicate, using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA) (24), in a Power Wave X spectrophotometer absorbance reader (Bio-Tek, Winooski, VT). The values were extrapolated by comparison with the standard curve for bovine serum albumin (Sigma) in the same diluents as the sample.

The peptidase activity was quantified based on the amount of β-naphthylamine (for APN) and 4-methoxy-β-naphthylamine (for DPPIV) released (25) as the result of incubation (30 min, 37 °C), in 96-well flat bottom microplates, recycled in ELX 50/8 washer (Bio-Tek), of 20 µl of plasma with substrate solution, prewarmed and diluted to 0.125 mmol/l (APN) or 0.2 mmol/l (DPPIV), in 0.05 mol/l of corresponding buffers containing 0.1 mg/ml bovine serum albumin. The naphthylamine or methoxy-β-naphthylamine content was estimated fluorometrically in a microplate fluorescence reader FL600FA (Bio-Tek) at 460/40 nm emission wavelength and 360/40 nm excitation wavelength. The fluorescence value obtained at zero time (blank) was subtracted and the relative fluorescence was then converted to picomoles of β-naphthylamine or methoxy-β-naphthylamine by comparison with their respective standard curves (β-naphthylamine or methoxy-β-naphthylamine, Sigma, dissolved in the same diluent as that used in the incubation). The peptidase activity was expressed as picomoles of hydrolyzed substrate per min per milligram of protein. The existence of a linear relationship between hydrolysis time and protein content in the fluorometric assay was a prerequisite.

The following substrates and conditions were used:

APN, L-Ala-β-naphthylamide (Sigma) (solubilized in 0.012 N HCl) in phosphate buffer, pH 7.4, containing 1 mmol/l of DL-dithiothreitol (Sigma) in the presence and absence of 0.02 mmol/l of puromycin (Sigma). Thus, two different APN activities were measured: that resulting from the assay in the presence of puromycin (puromycin insensitive activity (APM)) and that resulting from the subtraction of values obtained in the presence of puromycin from those obtained in the absence of puromycin (puromycin sensitive activity (PSA)).

DPPIV, H-Gly-Pro-4-methoxy-β-naphthylamide (Peninsula Laboratories, San Carlos, CA) (solubilized in dimethyl-sulfoxide) (Sigma) in Tris–HCl buffer, pH 8.3, in the presence and absence of 0.1 mmol/l of diprotin A (Bachem Bioscience, King of Prussia, PA). Thus, two different DPPIV activities were measured: that resulting from the assay in the presence of diprotin A (diprotin A insensitive activity (DPPIV-DI)) and that resulting from the subtraction of values obtained in the presence of diprotin A from those obtained in the absence of diprotin A (diprotin A sensitive activity (DPPIV-DS)).

Data analysis

The quantitative data were shown as mean ± s.e.m. and statistically analyzed using the software programs GraphPad Instat (GraphPad Software, La Jolla, CA), GraphPad Prism (GraphPad Software), and Prism 3.0 (Nextrails, San Ramon, CA). Regression analysis was performed in order to obtain standard curves for bovine serum albumin, β-naphthylamine, and methoxy-β-naphthylamine, and to evaluate the linear correlation. The Student's *t*-test was used to compare pairs of values. In all calculations, a minimum critical level of $P < 0.05$ was set.

RESULTS

Table 1 shows that the MSG and MSG-FD treatments induced a significant decrease in body mass, compared to C. The naso-anal length of MSG and MSG-FD was lower than C. The Lee index was higher in MSG and MSG-FD and lower in C-FD, in relation to C. There was an increase in mass of retroperitoneal and periepididymal fat pads relative to body mass in MSG and MSG-FD, compared to C. The glycemia levels showed no significant differences between the treatments.

Figure 2 shows the influence of puromycin on hydrolysis of L-Ala-β-naphthylamide (total APN) and H-Gly-Pro-4-methoxy-β-naphthylamide (total DPPIV) substrates by plasma. From the data shown in **Figure 2** the following enzyme activities were distinguished in this study: PSA for puromycin sensitive APN activity, and APM for puromycin insensitive

Table 1 Biometric parameters and glycemia in normal control (C), normal animals submitted to food deprivation (C-FD), monosodium glutamate-obese (MSG) and monosodium glutamate obese and food-deprived animals (MSG-FD)

	C (7)	C-FD (7)	MSG (8)	MSG-FD (7)
Body mass (g)	370 ± 14.65	339 ± 14.2	325 ± 9.45*	292 ± 10.25**
Naso-anal length (cm)	23.6 ± 0.4	24.2 ± 0.2	20.8 ± 0.2 ***	19.5 ± 0.2***
Lee index (g/cm)	0.29 ± 0.0027	0.28 ± 0.0055*	0.32 ± 0.0035***	0.33 ± 0.0055***
Periepididymal fat (% relative to body mass)	1.47 ± 0.23	1.18 ± 0.07	2.16 ± 0.20*	1.94 ± 0.16
Retroperitoneal fat (% relative to body mass)	0.88 ± 0.13	0.87 ± 0.07	1.83 ± 0.17**	1.70 ± 0.17**
Glycemia (mg/dl)	99.1 ± 9.0	84.3 ± 9.7	110 ± 14.5	88.6 ± 9.4

Values are mean ± s.e.m. Number of animals in parenthesis. Values in each column were obtained from the same animals.
MSG, monosodium glutamate.
***$P < 0.0001$, **$P < 0.004$, and *$P < 0.05$, compared to C (unpaired two-side Student's t-test).

Table 2 Kinetic parameters of PSA and APM neutral aminopeptidase, and diprotin sensitive (DPPIV-DS) and insensitive (DPPIV-DI) DPPIV activities obtained by fluorometric measurements of hydrolysis of naphthylamide derivative substrates by plasma from normal rats

Substrate β-naphthylamide	Enzyme	V_{max} (pmoles·min^{-1}·mg protein^{-1})	K_{cat} (s^{-1})	$K_m \times 10^{-5}$ (mol/l)	$K_{cat}/K_m \times 10^{-5}$ (mol/l^{-1}·s^{-1})
Ala-	PSA	203 ± 12	3.4	7.8 ± 1.1	0.43
Ala-	APM	1,014 ± 172*	17***	21.6 ± 2.6*	0.78***
H-Gly-Pro-4-methoxy-	DPPIV-DS	226 ± 18	3.8	0.24 ± 0.07	15.6
H-Gly-Pro-4-methoxy-	DPPIV-DI	1,575 ± 232**	26.2***	7.04 ± 0.5***	3.72***

Values are mean ± s.e.m (five and three animals respectively for calculations of DPPIV-DS and DPPIV-DI, and PSA and APM). Values for K_{cat} (maximum amount of substrate (picomoles) converted per second per enzyme unit (UI)) were calculated considering 1 UI = amount of enzyme, in 1 mg of protein, which hydrolyses 1 picomole of substrate per second. Kinetic analysis was performed by Michaelis–Menten methodology, using GraphPad Prism software program.
APM, puromycin insensitive; DPPIV, dipeptidyl peptidase IV; PSA, puromycin sensitive.
Comparisons of hydrolyzing activities on the same substrate with or without inhibitor (unpaired two-side Student's t-test): *$P < 0.01$, **$P < 0.0005$, ***$P < 0.0001$.

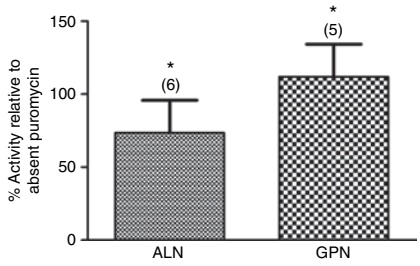

Figure 2 Effect of puromycin on the hydrolysis of synthetic substrates of neutral aminopeptidase (ALN) and DPPIV (GPN) by plasma of normal control animals. Values are mean ± s.e.m. Values without puromycin = 100%. Final concentration of puromycin in incubates = 0.02 mmol/l. Number of animals in parenthesis. *$P < 0.001$ in relation to value obtained without puromycin (paired one-side Student's t-test). ALN, L-Ala-β-naphthylamide; DPPIV, dipeptidyl peptidase IV; GPN, H-Gly-Pro-4-methoxy-β-naphthylamide.

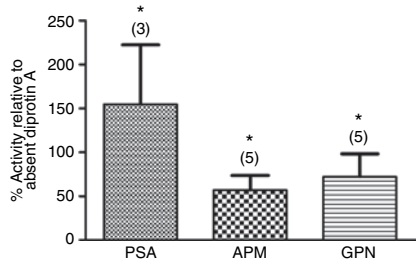

Figure 3 Effect of diprotin A on activities of PSA and APM neutral aminopeptidases and on the hydrolysis of synthetic substrate of DPPIV (GPN) by plasma of normal control animals. Values are mean ± s.e.m. Values without diprotin A = 100%. Final concentration of diprotin A in incubates = 0.016 mmol/l. Number of animals in parenthesis. *$P < 0.02$ in relation to value obtained without diprotin A (paired one-side Student's t-test). APM, puromycin insensitive; DPPIV, dipeptidyl peptidase IV; GPN, H-Gly-Pro-4-methoxy-β-naphthylamide; PSA, puromycin sensitive.

APN activity. Puromycin also induced a slight increase in the hydrolysis of the H-Gly-Pro-4-methoxy-β-naphthylamide substrate by plasma. Figure 3 shows the effect of diprotin A on plasma PSA and APM as well as on hydrolysis of H-Gly-Pro-4-methoxy-β-naphthylamide (total DPPIV) by plasma. Diprotin increased plasma PSA activity, while decreasing APM activity. Diprotin also decreased the hydrolysis of the H-Gly-Pro-4-methoxy-β-naphthylamide by plasma. Based on the profile of hydrolysis of this substrate in the presence of diprotin A, the following enzyme activities was distinguished in this study: DPPIV-DS for diprotin A sensitive DPPIV activity, and DPPIV-DI for diprotin A insensitive DPPIV activity. The kinetic features distinguishing plasma PSA, APM, DPPIV-DS, and DPPIV-DI are shown in Table 2.

Table 3 shows that the level of plasma activity of APM in C was higher than that of PSA, while that of DPPIV-DI was higher than that of DPPIV-DS. There was no significant difference in plasma PSA, APM, and DPPIV-DS activities from the

Table 3 Effect of obesity and food deprivation on plasma peptidase activity (UP·mg/protein).

	PSA	APM	DPPIV-DS	DPPIV-DI
C	425.74 ± 167.02 (4)	777.83 ± 234.27 (6)	364.12 ± 81.41 (5)	1,533.57 ± 178.75 (4)
C-FD	144.69 ± 37.99 (3)	377.58 ± 58 (6)	421.92 ± 150.24 (4)	1,879.47 ± 149.5 (5)
MSG	159.99 ± 34.48 (4)	691.81 ± 186.54 (7)	320.33 ± 74.18 (5)	1,969.84 ± 256.87 (5)
MSG-FD	249.21 ± 53.56 (3)	302.7 ± 27.15 (5)	501.51 ± 85.75 (4)	1,019.46 ± 95.65* (5)

Values are mean ± s.e.m. Number of animals in parenthesis.
APM, puromycin insensitive neutral aminopeptidase; C, normal control animals; C-FD, normal animals submitted to food deprivation; DPPIV-DI, diprotin insensitive dipeptidyl peptidase IV; DPPIV-DS, diprotin sensitive dipeptidyl peptidase IV; MSG, monosodium glutamate-obese animals; MSG-FD, monosodium glutamate obese and food-deprived animals; PSA, puromycin sensitive neutral aminopeptidase; UP, picomoles of hydrolyzed substrate per minute.
*$P < 0.04$ in relation to C (unpaired two-side Student's *t*-test).

Table 4 Correlation among enzyme activities and biometric parameters in normal control (C), normal animals submitted to food deprivation (C-FD), monosodium glutamate obese (MSG) and monosodium glutamate obese and food-deprived animals (MSG-FD)

	C	C-FD	MSG	MSG-FD
Body mass				
APM	A	s = –0.227 ± 0.076 $P < 0.05$; $r^2 = 0.70$	A	A
DPPIV-DI	A	A	s = 0.016 ± 0.0046 $P < 0.03$; $r^2 = 0.75$	s = 0.053 ± 0.0088 $P < 0.002$; $r^2 = 0.88$
Naso-anal length				
PSA	A	s = 0.0031 ± 0.0010 $P < 0.03$; $r^2 = 0.66$	s = 0.0006 ± 0.0002 $P < 0.04$; $r^2 = 0.53$	A
Lee index				
APM	A	s = –0.00009 ± 0.00002 $P < 0.02$; $r^2 = 0.80$	A	A
DPPIV-DI	A	A	A	s = 0.0003 ± 0.00000006 $P < 0.008$; $r^2 = 0.79$
Periepididymal fat				
DPPIV-DI	A	A	A	s = 0.002 ± 0.0004 $P < 0.003$; $r^2 = 0.87$
Retroperitoneal fat				
APM	A	s = –0.003801 ± 0.0012 $P < 0.04$; $r^2 = 0.70$	A	A
PSA				
DPPIV-DS	A	s = –1.393 ± 0.213 $P < 0.008$; $r^2 = 0.93$	A	A

Linear correlation analyses were performed by Prisma 3.0 software program on paired biometric parameters and glycemia vs. peptidase activities and on paired peptidase activities in different treatments. The parameters that did not show any correlation were not presented.
A, absent correlation; APM, puromycin insensitive neutral aminopeptidase; DPPIV-DI, diprotin insensitive dipeptidyl peptidase IV; DPPIV-DS, diprotin sensitive dipeptidyl peptidase IV; P, probability; PSA, puromycin sensitive neutral aminopeptidase; r^2, coefficient of determination; s, slope.

different treatments (C-FD, MSG, and MSG-FD) in relation to C. DPPIV-DI activity decreased about 1.5-fold in plasma from MSG-FD.

Table 4 shows the results of the search for significant correlations by linear regression analyses of all paired combinations of the biometric parameters and glycemia vs. peptidase activities, and between paired peptidase activities. In normal animals, there was no correlation between any pairs of parameters. In the MSG animals, there was a correlation between DPPIV-DI vs. body mass (positive) and PSA vs. naso-anal length (positive). Food deprivation in the normal animals determined correlations between APM vs. body mass (negative), PSA vs. naso-anal length (positive), APM vs. Lee index (negative), APM vs. retroperitoneal fat pad, and PSA vs. DPPIV-DS (negative). In the MSG animals, food deprivation determined positive correlations between DPPIV-DI vs. body mass, DPPIV-DI vs. Lee index and DPPIV-DI vs. periepididymal fat pad.

DISCUSSION

As expected, nonfasted and 72-h fasted MSG obese rats show high adiposity with low muscle mass and normoglycemia (26,27).

The sensitivity to diprotin A and different kinetic properties distinguished, for the first time, two DPPIV activities in rat

plasma: DPPIV-DS and DPPIV-DI. As shown by the V_{max} values, DPPIV-DI was about seven times more active than DPPIV-DS. However, the affinity of DPPIV-DS was higher (lower K_m) than that of DPPIV-DI. Furthermore, the K_{cat} of DPPIV-DI was about seven times higher than that of DPPIV-DS, but this relatively high K_{cat} was not followed by an increment in catalytic efficiency (K_{cat}/K_m). As occurs in the central nervous system (28,29), the sensitivity to puromycin distinguished two APN activities with peculiar kinetic parameters in rat plasma: PSA and APM. As shown by the V_{max} values, APM was about five times more active than PSA. However, the affinity of PSA was higher (lower K_m) than that of APM, but the K_{cat} of PSA was about five times lower than that of APM and this relatively low K_{cat} of PSA was followed by a decrease in catalytic efficiency (K_{cat}/K_m). It is noteworthy that the correlations and changes resulting from the treatments under study were mainly related to APM and DPPIV-DI, and that those unchanged (DPPIV-DS and PSA) were correlated among themselves in normal food-deprived animals. Despite the lack of effect of the treatments on the absolute levels of PSA and APM, the correlations of these activities with several other parameters under study are suggestive. For example, in the case of APM, the negative correlation with the retroperitoneal fat pad in normal food-deprived animals suggests that the high plasma level of growth hormone found during food deprivation (30) is related to the modulation (downregulation) of the levels of circulating somatostatin by this enzyme. In addition to the modulation by APM, a positive correlation between naso-anal length and PSA in normal food-deprived animals suggests that this enzyme is also involved in somatostatin regulation, because the increase in its activity levels is able to reduce the levels of somatostatin (resulting in increased plasma levels of growth hormone). The mechanism of regulating plasma levels of growth hormone by this enzyme activity does not seem to be activated in animals with normal energy status (normal control animals), because there was no correlation between retroperitoneal fat pad and this plasma enzyme in normal control animals, suggesting the existence of a threshold triggering the mechanism for regulation of growth hormone levels linked to the duration of starvation. The experimental model of obesity used in this study is known to cause hypothalamic lesion, which results in a reduced release of growth hormone-releasing hormone and a subsequent drop in the secretion of growth hormone (31,32). DePaolo and Steger (33) report that in this model of obesity, compared to normal animals, the hypothalamic and plasma somatostatin concentrations are reduced, whereas they are increased in the gastro-entero-hepatic region, and even with replacement of growth hormone plasma levels of somatostatin, did not normalize. Because there was no correlation of APM with any of the biometric parameters under study (retroperitoneal and periepididymal fat pads, body mass and Lee index) in MSG animals, it is possible that regulation of somatostatin levels by APM is disabled in the obese animals.

The decrease in DPPIV-DI activity in the food-deprived MSG animals, in relation to the controls, adds one more piece of evidence concerning the role of this catalytic activity in metabolic disorders, and reinforces the important previous finding that mice lacking DPPIV are protected against obesity and insulin resistance (34). The present study also shows that DPPIV-DI was correlated with several biometric parameters in nonfasted MSG (body mass) and fasted MSG (body mass, periepididymal fat, and Lee index). Taken together, data obtained with the ablation of DPPIV (34), and those from the present study, both suggest a contribution of altered levels of plasma DPPIV in disrupted control of some peptides such as glucagon-like peptide-1 (9,18). Furthermore, DPPIV is known to hydrolyze NPY 1-36 (9,10,18), and has recently been linked to the control of cholecystokinin-8 levels (35). Thus its reduction in the food-deprived MSG obese animals could be related to the recovery of energy balance, for example, by opposing the anorexigenic effect due to the increase of fat adsorption-stimulated synthesis and secretion of cholecystokinin-8 (35), and also by contributing to the increased antilipolytic effect of NPY 1-36 (36) and preventing the angiogenic effect of NPY 3-36 (product of the hydrolysis of NPY 1-36 by DPPIV) (37).

In conclusion, correlations between plasma APM (predominant form of plasma APN) and biometric parameters were suggestive of downregulation of somatostatin in food-deprived rats, but not in MSG obese rats. Correlations between two forms of plasma DPPIV and biometric parameters and the decrease in DPPIV-DI (the predominant form of plasma DPPIV) suggest the role of this enzyme activity in restoring the energy balance in MSG obese rats during food deprivation. These data may provide useful information in the clinical evaluation of inhibitors of both enzymes as new drug candidates.

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DISCLOSURE

The authors declared no conflict of interest.

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