

# Insulin-regulated aminopeptidase in adipocyte is Cys-specific and affected by obesity

Rafaela Fadoni Alponi<sup>1,2,3</sup>, Luciana Godoy Viana<sup>1</sup>, Norma Yamanouye<sup>1</sup> and Paulo Flavio Silveira<sup>1,2</sup>

<sup>1</sup>Laboratory of Pharmacology, and <sup>2</sup>Unit of Translational Endocrine Physiology and Pharmacology, Instituto Butantan, Avenue Vital Brasil, 1500, CEP05503-900 Sao Paulo, Brazil

<sup>3</sup>Department of Physiology, Universidade de Sao Paulo, Sao Paulo, Brazil

Correspondence should be addressed to P F Silveira

**Email**  
paulo.silveira@butantan.gov.br

## Abstract

Insulin-regulated aminopeptidase (IRAP, EC 3.4.11.3) in adipocytes is well known to traffic between high (HDM) and low (LDM) density microsomal fractions toward the plasma membrane (MF) under stimulation by insulin. However, its catalytic preference for aminoacyl substrates with N-terminal Leu or Cys is controversial. Furthermore, possible changes in its traffic under metabolic challenges are unknown. The present study investigated the catalytic activity attributable to EC 3.4.11.3 in HDM, LDM and MF from isolated adipocytes of healthy (C), food deprived (FD) and monosodium glutamate (MSG) obese rats on aminoacyl substrates with N-terminal Cys or Leu, in absence or presence of insulin. Efficacy and reproducibility of subcellular adipocyte fractionation procedure were demonstrated. Comparison among HDM vs LDM vs MF intragroup revealed that hydrolytic activity trafficking from LDM to MF under influence of insulin in C, MSG and FD is only on N-terminal Cys. In MSG the same pattern of anterograde traffic and aminoacyl preference occurred independently of insulin stimulation. The pathophysiological significance of IRAP in adipocytes seems to be linked to comprehensive energy metabolism related roles of endogenous substrates with N-terminal cysteine pair such as vasopressin and oxytocin.

## Key Words

- ▶ aminopeptidases
- ▶ adipocyte
- ▶ obesity
- ▶ insulin
- ▶ diet

*Journal of Molecular Endocrinology*  
(2015) 55, 1–8

## Introduction

During the prandial period, insulin stimulates the translocation of an intracellular vesicle from low density microsomal (LDM) fraction to the plasma membrane (MF) of adipocytes (Watson & Pessin 2001, Zeyda & Stulnig 2009, Jordens *et al.* 2010, Dimitriadis *et al.* 2011). This vesicle contains glucose transporter type 4 (GLUT4) and insulin-regulated aminopeptidase (IRAP, EC 3.4.11.3) (Keller *et al.* 1995, Keller 2004, Jordens *et al.* 2010). Hydrolytic activity on angiotensin (Ang)-3 (Ruster & Wolf 2013), lysil-bradykinin (Ino *et al.* 2003), vasopressin (AVP) (Ino *et al.* 2003) and oxytocin (OXT) (Keller 2004),

as well Ang-4 receptor function (Albiston *et al.* 2010) have been attributed to EC 3.4.11.3. Several studies show variation of EC 3.4.11.3 protein expression in LDM, MF and also in compartment of cellular protein origin, corresponding to high density microsomal (HDM) fraction (Alberts *et al.* 2008), under different pathophysiological conditions (Maianu *et al.* 2001, Jordens *et al.* 2010). LDM consists of microsomes without ribosomes and with portions of endoplasmic reticulum (ER) and fragments of Golgi apparatus. HDM consists of microsomes with ribosomes adhered to its outer surface.

The inner side of microsomes is biochemically equivalent to the lumen of the ER (Alberts *et al.* 2008). However, concomitant hydrolytic activity of EC 3.4.11.3 has been rarely evaluated in these locations (Sano *et al.* 2005). In general, catalytic ability has been assessed only under basal conditions and using aminoacyl substrates with N-terminal Leu (Ross *et al.* 1996, Sano *et al.* 2005). Curiously, hydrolytic activity in aminoacyl substrates with N-terminal Cys has been also consensually attributed to IRAP based on rare experimental reports (Carrera-González *et al.* 2011). This consensus was experimentally reinforced by recent findings that hydrolytic activity of MF from adipocytes in aminoacyl substrate with N-terminal Cys prevails over that with N-terminal Leu (R F Alponti and P F Silveira, unpublished observations). On the other hand, disturbances related with insulin are critical in the development of various diseases (Gross *et al.* 2004, Tesouro & Cardillo 2011). For example, insulin resistance in muscle and adipose tissues is a very common occurrence in obese (Bonala *et al.* 2013, Lo *et al.* 2013).

This study aimed to identify whether intracellular traffic regulated by insulin in adipocytes from healthy (C), food deprived (FD), and monosodium glutamate (MSG) obese rats is preferentially attributable to EC 3.4.11.3 hydrolytic activity on N-terminal Cys or Leu.

## Experimental procedures

### Animals and treatments

Immediately after birth, male Wistar rats were housed with a lactant female in a polypropylene box (inside length  $\times$  width  $\times$  height = 56 cm  $\times$  35 cm  $\times$  19 cm) within a ventilated container (Alesco Ind. Com Ltda, Monte Mor, SP, Brazil) under controlled temperature ( $24 \pm 2^\circ\text{C}$ ), relative humidity ( $65 \pm 1\%$ ) and 12 h light:12 h darkness photoperiod (lights on at 0600 h). Twenty-four hours after birth, the animals were subdivided into two groups, treated according to the methodology described by Alponti & Silveira (2010). The i-MSG group received a daily s.c. bolus injection of L-glutamic acid monosodium salt (Sigma) in saline 0.9% (4 mg/g body mass), in the cervical region, between 0730 and 0900 h of light period (1330–1500 h), at a maximum volume of 0.2 ml, until they were 10 days old. The i-control group received the same volume of saline 0.9% s.c., in the same scheme as previously described. On the 22nd day, the animals were weaned and the female was removed from the cage. On the 90th day, obesity was evaluated by Lee index, calculated by body mass (g)<sup>0.33</sup>/naso-anal length (cm)

(Nakagawa *et al.* 2000, Kaufhold *et al.* 2002). Thus, obese (MSG) animals were selected from i-MSG group by Lee index  $>0.300$  and the i-control rats were subdivided into two groups: control (Lee index  $\leq 0.300$ ) and FD control (control animals fasted for 72 h). Food deprivation was performed by transferring pairs of animals into metabolic cages without food for 72 h. Drinking water *ad libitum* was available all the time.

The conducts and procedures involving animal experiments were approved by the Butantan Institute Committee for Ethics in Animal Experiments (License number CEUAIB 684/2009) in compliance with the recommendations of the National Council for the Control of Animal Experimentation of Brazil (CONCEA). All efforts were made to minimize suffering.

### Adipocytes isolation

After euthanasia by decapitation, the retroperitoneal fat pad was removed through manual dissection and washing with saline 0.9%. Total mass (g) of fat pad was measured, 3 g was separated and submitted to collagenase digestion as described by Rodbell (1964). Briefly, this quantity of fat pad was added to 9 ml of DMEM (Cultilab, Campinas, SP, Brazil) containing 25 mM HEPES (pH 7.5), 4% BSA (Sigma) and 45 mg of collagenase (Sigma), and then incubated at  $37^\circ\text{C}$ , for 1 h, under gentle shaking. Subsequently, this mixture was washed with eight volumes of buffered washing solution (115 mM NaCl, 0.8 mM  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 5.3 mM KCl, 1.4 mM  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.89 mM  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , 25 mM HEPES, 1 mM Na pyruvate, 145 mM BSA; pH 7.4, at  $25^\circ\text{C}$ ), and then filtered through a nylon mesh. This filtrate was centrifuged at 13 g for 1 min, at  $25^\circ\text{C}$ . The pellet containing vascular stroma (capillaries, endothelial cells, mast cells, macrophages and epithelial cells) was removed by suction and discarded, while the supernatant, containing the suspension of adipocytes, was washed and centrifuged again at the same conditions three more times. The resultant suspension of isolated adipocytes was microscopically observed to verify the absence of vascular stroma.

### Incubation of isolated adipocytes with insulin

KRBH-BSA buffer (121 mM NaCl, 1.2 mM  $\text{MgSO}_4$ , 4.9 mM KCl, 2.4 mM  $\text{NaH}_2\text{PO}_4$ , 0.33 mM  $\text{CaCl}_2$ , 20 mM HEPES, pH 7.4, 4% BSA, 5 mM glucose) was added to adipocytes suspension obtained from each animal, at a ratio of 3 ml of buffer for each gram of adipose tissue that originated the suspension. Subsequently, saline or  $10^{-6}$  M insulin (Sigma) at a maximum volume of 60  $\mu\text{l}$  were added and

then the mixture was incubated for 15 min at 37 °C under gentle agitation. Insulin was dissolved in water and diluted in KRBH-BSA buffer. After the incubation period, the mixture was immediately used in the next procedure.

### Obtaining HDM, LDM and MF

Based on McKeel & Jarett (1970), the suspension of adipocytes isolated from 3 g of fat pad from each animal was put in 2.3 ml homogenization buffer (20 mM Tris-HCl/255 mM sucrose, pH 7.4, 20 °C) (HB) and sonicated for 20 s at amplitude of 20  $\mu$ m at 20 kHz. The resulting suspension was then centrifuged at 2000 *g*, for 10 min and the top layer containing solidified fat cake was discarded, and the infranatant was collected. Subsequently, 0.1% (relative to final volume) Triton X-100 were added to this infranatant, which was homogenized in a potter glass at 800 r.p.m., for 3 min and centrifuged at 2000 *g*, for 10 min. The new top layer of remaining fat was discarded, and the infranatant was collected. Afterward 2.3 ml HB was added to this infranatant, which was homogenized by ten strokes with the teflon pestle in a potter glass and then ultracentrifuged (Hitachi model HIMAC CP60E) (16 000 *g* for 15 min). Again, the new top layer formed was carefully removed and discarded. The intermediate layer (CI-1) was removed and reserved for subsequent preparation of high and LDM fractions. The pellet (P-1) was resuspended in 2.3 ml HB and ultracentrifuged again at the same conditions (16 000 *g* for 15 min). The supernatant was discarded, the pellet (P-2) was resuspended in 2 ml HB and carefully transferred to another tube on a layer of sucrose solution (1.12 M sucrose containing 20 mM Tris-HCl previously applied to the bottom of the tube) and, thus, ultracentrifuged (101 000 *g* for 70 min). The intermediated layer (CI-2) formed was collected and resuspended in 1.9 ml HB and ultracentrifuged (48 000 *g* for 45 min). After this ultracentrifugation, the pellet (P-3) formed was resuspended in 1.9 ml HB and ultracentrifuged again (48 000 *g* for 45 min). The new pellet (P-4) containing plasma MF was solubilized in 1.9 ml HB containing 0.1% Triton X-100. Previously reserved CI-1 was ultracentrifuged (48 000 *g* for 20 min). The pellet (P-5) obtained was reserved for preparation of HDM fraction and the supernatant containing LDM fraction was ultracentrifuged (180 000 *g* for 70 min). The pellet formed (P-6) from this last ultracentrifugation was resuspended in 2.5 ml HB and ultracentrifuged again at the same conditions. The new pellet thus obtained contains LDM fraction (LDM) and was resuspended in the same volume with HB containing 0.1% Triton X-100. Previously

reserved P-5 was resuspended in 2.5 ml HB and ultracentrifuged (48 000 *g* for 20 min). The new pellet thus obtained contains HDM fraction and was resuspended in the same volume (2.5 ml) with HB containing 0.1% Triton X-100. All procedures were carried out at 4 °C. Plasma MF, and high and LDM fractions were immediately frozen on dry ice and stored at -80 °C until their use.

### Protein

Protein content (HDM, LDM and MF) was measured in triplicate at 630 nm in a Bio-Tek FL600FA microplate fluorescence/absorbance reader (BioTek, Winooski, VT, USA) by Bradford method (Bradford 1976) with a Bio-Rad protein assay kit (Bio-Rad) using BSA dissolved in the same sample diluent as standard.

### Two-dimensional gel electrophoresis

Protein samples (80  $\mu$ g) from HDM, LDM and FM obtained from the same animal were first individually precipitated with acetone (Jiang *et al.* 2004). According to the methodology described by Luna *et al.* (2013), each pellet thus obtained was suspended in 125  $\mu$ l of DeStreak rehydration solution and 0.5% immobilized pH gradient (IPG) buffer pH 3-10 (GE Healthcare, Uppsala, Sweden). IPG strips of 7 cm, pH range 3-10, were passively rehydrated overnight at room temperature. Isoelectric focusing (IEF) was performed on an Ettan IPGphor II (GE Healthcare) using a program for a total of 5952 Vh according to the manufacturer's suggestion, including an initial step at low voltage of 100 V for 2 h to allow salt to migrate out of the strip. After IEF, the strips were initially incubated with equilibration buffer (75 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue) containing 1% dithiothreitol (DTT) for 15 min. Next, solutions were discarded and the strips were incubated in an equilibration buffer containing 2.5% iodoacetamide for 15 min at room temperature. After equilibration, the strips were applied to the second dimension to separate proteins on a 12.5% SDS-PAGE and run on an Mini-PROTEAN Tetra electrophoresis system (Bio-Rad) according to the manufacturer's instructions. After running the gels, they were fixed (5% acetic acid, 20% methanol) for at least 30 min and stained with Coomassie Brilliant Blue G for 3 days. The stained gels were scanned on ImageScanner III (GE Healthcare). The identity and densitometry of the well-stained spots were analyzed in digitized images (8-bit grayscale, 300 dpi) by using the ImageMaster 2D Platinum v7.0 Software

(GE Healthcare). Biological samples of three healthy animals were analyzed.

### Peptidase activities

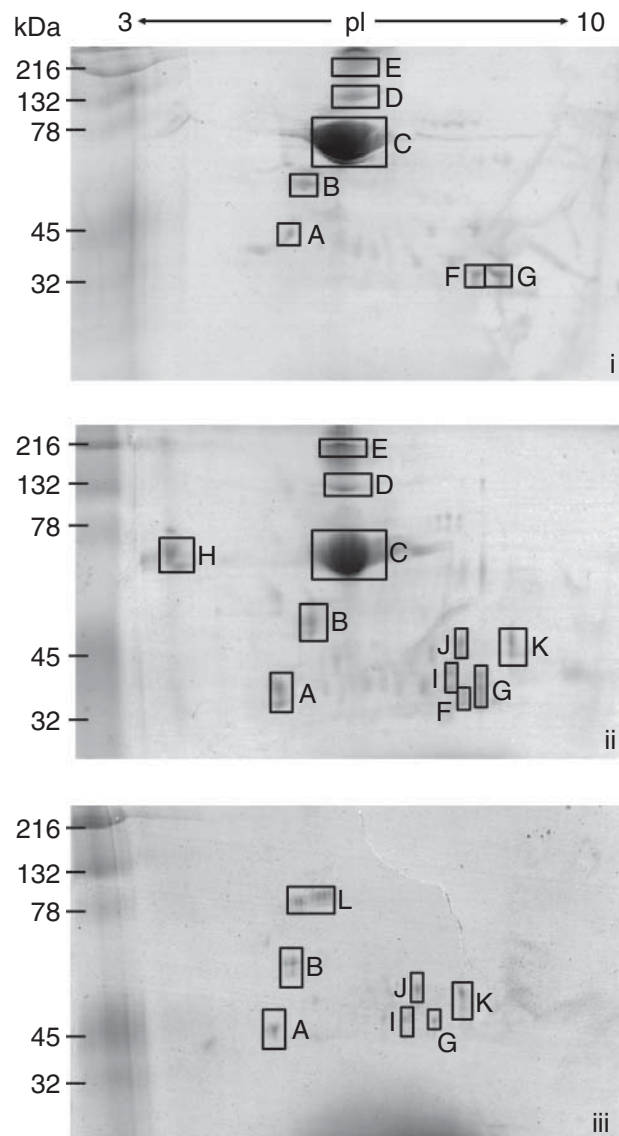
Cystyl-aminopeptidase (CysAP) and leucyl-aminopeptidase (LeuAP) activities were quantified based on the amount of  $\beta$ -naphthylamine released (Gaspardo-Clemente *et al.* 2003) as the result of incubation (180 min, 37 °C), in 96-well flat-bottom microplates, of adipocyte fractions (HDM, LDM, MF), in triplicate, with prewarmed solution of 1.5 mM L-Cys-di- $\beta$ -naphthylamide (Sigma) (solubilized in 0.012 N HCl) in Tris maleate buffer 0.05 M, pH 6.0, with BSA 0.1 mg/ml (CysAP activity) or 1.5 mM Leu- $\beta$ -naphthylamide (Sigma) (solubilized in methanol) in phosphate buffer 0.05 M, pH 7.4, with BSA 0.1 mg/ml, 1 mM DL-DTT (LeuAP activity). The  $\beta$ -naphthylamine content was estimated fluorometrically (with a Bio-Tek FL600FA microplate fluorescence reader) at 415 nm emission wavelength and 335 nm excitation wavelength. The fluorescence value obtained at zero time (blank) was subtracted, and the relative fluorescence was then converted to picomoles of  $\beta$ -naphthylamine by comparison with the standard curve of  $\beta$ -naphthylamine (Sigma) dissolved in the same diluent used in the incubation. CysAP and LeuAP activities were expressed as picomoles of hydrolyzed substrate per minute per milligram of protein. The existence of a linear relationship between hydrolysis time and protein content in the fluorometric assay was a prerequisite.

### Data analysis and results presentation

The quantitative data were shown as mean  $\pm$  S.E.M. and statistically analyzed using the software GraphPad Prism (GraphPad, La Jolla, CA, USA). Regression analysis was performed in order to obtain standard curves of protein and  $\beta$ -naphthylamine. ANOVA, followed by multiple comparison Tukey test, when differences were detected, was used to compare the values among three subcellular fractions. Unpaired two-side Student's *t*-test were used to compare values between two subcellular fractions. In all the calculations, a minimum critical level of  $P < 0.05$  was set.

### Results

Simple visual inspection of the gels evidenced similar profiles in the same fraction among the different animals. Representative gel images of these profiles (Fig. 1) show



**Figure 1**

Representative 2-DE gel images of (i) high (HDM) and (ii) low (LDM) density microsomal and (iii) plasma membrane (MF) fractions from isolated adipocytes of three healthy animals. Samples (80  $\mu$ g) were applied to 3–10 IPG strips followed by electrophoresis on 12.5% acrylamide gels. Spots are designated A to L.

that well-stained protein spots had molecular mass (MM) and isoelectric point (pI) respectively ranging between 34–225 kDa and 4.96–7.79 for HDM; 34–225 kDa and 3.56–8.00 for LDM; and 37–92 kDa and 4.96–8.00 for MF. HDM presented four spots (C, D, E and F) shared with LDM, being only the spot C with lower density than in LDM (Table 1). In addition to these four spots shared with HDM, LDM presented one peculiar (H) and three others spots shared with MF (I, J and K), two (I and K) with higher

**Table 1** Comparison of well-stained protein spots in two-dimensional gel electrophoresis (2-DE) images. Values are means  $\pm$  s.e.m. of three healthy animals

SPOT	M.M (kDa)	pI	Density		
			HDM	LDM	FM
A	39	4.96	0.078 $\pm$ 0.013 <sup>a</sup>	0.212 $\pm$ 0.015 <sup>b</sup>	0.064 $\pm$ 0.011 <sup>a</sup>
B	56	5.31	0.073 $\pm$ 0.013 <sup>a</sup>	0.195 $\pm$ 0.029 <sup>b</sup>	0.066 $\pm$ 0.001 <sup>a</sup>
C	72	5.81	0.354 $\pm$ 0.073	0.836 $\pm$ 0.096*	none
D	143	5.83	0.093 $\pm$ 0.010	0.152 $\pm$ 0.046	none
E	225	5.82	0.104 $\pm$ 0.041	0.323 $\pm$ 0.163	none
F	34	7.48	0.101 $\pm$ 0.006	0.069 $\pm$ 0.019	none
G	37	7.79	0.090 $\pm$ 0.022	0.144 $\pm$ 0.041	0.049 $\pm$ 0.001
H	74	3.56	none	0.210 $\pm$ 0.071	none
I	44	7.31	none	0.143 $\pm$ 0.024	0.041 $\pm$ 0.011*
J	46	7.42	none	0.175 $\pm$ 0.063	0.089 $\pm$ 0.005
K	45	8	none	0.242 $\pm$ 0.063	0.049 $\pm$ 0.002*
L	92	5.84	none	none	0.147 $\pm$ 0.080

None, absent spot; M.M, molecular mass; pI, isoelectric point; HDM, high density microsomal; LDM, low density microsomal; MF, plasma membrane. HDM vs LDM vs MF (ANOVA,  $P < 0.05$ ; multiple comparison Tukey test,  $P < 0.05$ , different letters (<sup>a,b</sup>) indicate significant differences of spot density among subcellular fractions). When one spot was absent in any fraction, the two others fractions were compared using the unpaired two-side Student's *t*-test ( $*P < 0.05$ ).

density than in MF (Table 1). MF presented one peculiar spot (L). The spots A, B and G were present in HDM, LDM and MF, being A and B with higher density in LDM than in HDM and MF (Table 1).

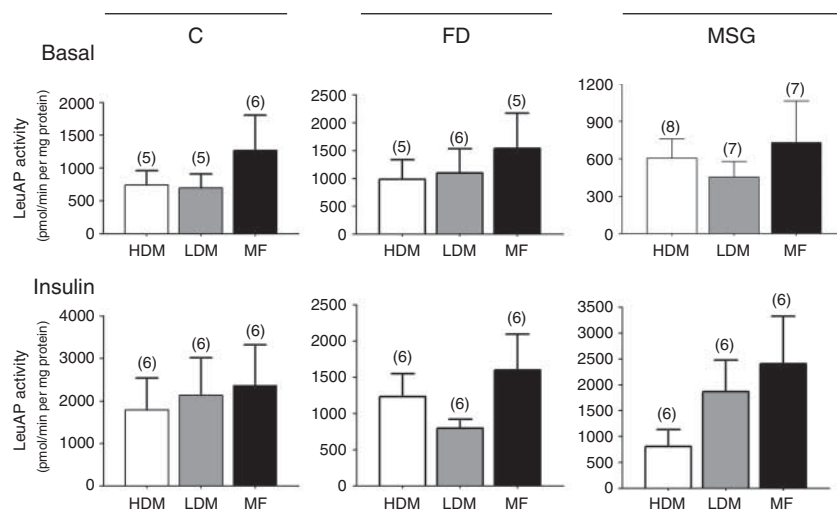
Figure 2 shows no differences of LeuAP activity among HDM, LDM and from C, FD and MSG under basal or insulin-stimulated conditions.

Figure 3 shows no differences of CysAP activity among HDM, LDM and MF from C and FD under basal condition. However, under basal condition this activity in MSG was

higher in MF than in HDM and LDM. After insulin stimulation, CysAP activity in MSG remained higher in MF and became also higher in MF than in HDM and LDM in C and FD.

## Discussion

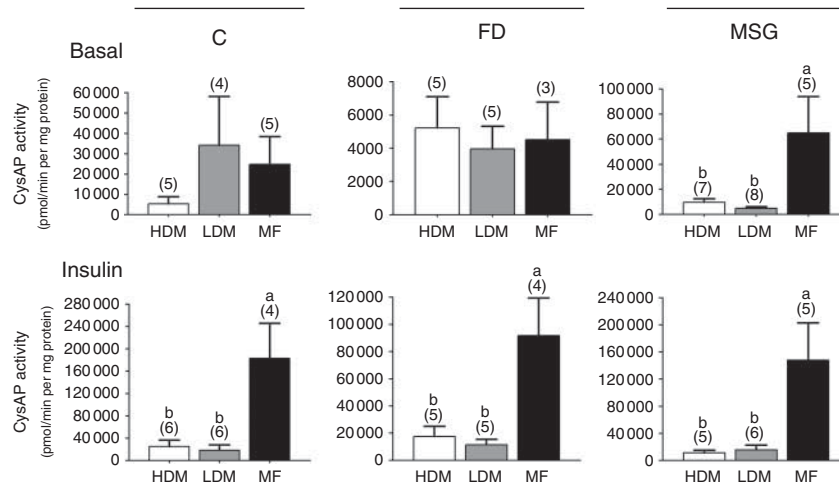
By comparing the two-dimensional gel electrophoresis (2-DE) spot patterns from different samples, changes in individual proteins can be detected and quantified.

**Figure 2**

Leucyl-aminopeptidase (LeuAP) activity of high (HDM) and low (LDM) density microsomal and plasma membrane (MF) fractions from isolated adipocytes of retroperitoneal fat pad of control (C), food deprived (FD) and obese (MSG) rats in basal condition or under insulin stimulation. Values are

mean  $\pm$  s.e.m. from triplicates of *n* animals (*n* in parenthesis over the bars). ANOVA, HDM vs LDM vs MF: basal (C,  $P = 0.5125$ ; FD,  $P = 0.7072$ ; MSG,  $P = 0.6882$ ); insulin (C,  $P = 0.8962$ ; FD,  $P = 0.2894$ ; MSG,  $P = 0.2511$ ).





**Figure 3**

Cystyl-aminopeptidase (CysAP) activity of high (HDM) and low (LDM) density microsomal and plasma membrane (MF) fractions from isolated adipocytes of retroperitoneal fat pad of control (C), food deprived (FD) and obese (MSG) rats in basal condition or under insulin stimulation. Values are mean  $\pm$  s.e.m. from triplicates of  $n$  animals ( $n$  in parenthesis over the bars).

ANOVA, HDM vs LDM vs MF: basal (C,  $P=0.3973$ ; FD,  $P=0.8675$ ; MSG,  $P=0.0095$ ); insulin (C,  $P=0.0031$ ; FD,  $P=0.0049$ ; MSG,  $P=0.0118$ ). Multiple comparison Tukey test ( $P<0.05$ , different letters in the same group and stimulus indicate significant differences).

This also allows identification of proteins that are characteristic of a specific subcellular fraction. In the present study, subcellular adipocyte fractionation in different animals resulted in a similar protein pattern of each fraction. In addition, different band fingerprinting of each fraction obtained by 2-DE procedure assured that each fraction contained different protein patterns, thus demonstrating that the enzyme assays were not cross-contaminated due to mixture of fractions. The effectiveness of this subcellular adipocyte fractionation was previously demonstrated only by the use of markers as cytochrome C reductase for HDM, galactosyltransferase for LDM, and 5'-nucleotidase for MF (Simpson *et al.* 1983, Maianu *et al.* 2001).

IRAP is a member of the M1 family of zinc-dependent metalloproteinases (Maianu *et al.* 2001, Ye *et al.* 2008) that has been widely studied in CNS and adipocytes (Keller *et al.* 1995, Keller 2004, Albiston *et al.* 2010, Jordens *et al.* 2010, Bogan 2012). In adipocytes, the regulation of its traffic from LDM toward MF by insulin (Keller *et al.* 1995, Keller 2004, Jordens *et al.* 2010) allows catalytic or receptor roles of this protein in extracellular environment. Most of the literature reports in this sense assess this traffic by immunoblotting or immunofluorescence (Jordens *et al.* 2010, Hirata *et al.* 2011, Bogan 2012) without concomitant evaluation of catalytic activity. When catalytic activity is measured, aminoacyl substrates with N-terminal Leu are usually used (Lew *et al.* 2003, Saníger *et al.* 2011). The present study showed only a trend, without statistical

significance, for insulin-stimulated traffic of LeuAP activity in adipocytes, which did not change as a function of metabolic challenges applied. However, insulin effectively stimulates CysAP activity trafficking from LDM toward MF in all animal groups under study. In this way, it fits in the current functional concept of IRAP. It was also noteworthy that the metabolic challenge posed by MSG obesity interferes directly on CysAP activity, influencing its anterograde traffic, independently of insulin stimulation. The insulin-dependent traffic of CysAP activity has important pathophysiological implications, since endogenous substrates with N-terminal cysteine pair susceptible to hydrolysis by this enzyme activity, that are available in the extracellular environment, have consistent and comprehensive roles related to energy metabolism. One substrate is AVP, which plays an antipolytic role in adipocyte (Hiroshima *et al.* 2007), besides anti-diuretic, vasoconstrictor (Küchler *et al.* 2010) and a memory facilitator agent (Ramanathan *et al.* 2012). Another is OXT, best known for its role in lactation and parturition (Caldwell *et al.* 1990), but also known as an anorexigenic agent, reducing meal size and intake of highly palatable foods, such as sucrose (Maejima *et al.* 2011) and increasing latency to the first meal (Blevins & Ho 2013). In addition, OXT has been recognized as a facilitator of interpersonal attraction (Scheele *et al.* 2012, 2013), providing relaxed physiological states (Carter & Porges 2013). Thus, insulin-independent stimulation of

CysAP activity traffic from LDM toward MF is a deleterious dysregulation in MSG obesity, since it facilitates extracellular hydrolytic action of IRAP normally under insulin control on peptides whose actions contribute to homeostasis.

In conclusion, catalytic preference and insulin-regulated traffic of IRAP in adipocytes that is disturbed by obesity are interrelated with comprehensive roles of endogenous substrates with N-terminal cysteine pair in energy metabolism homeostasis.

#### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

#### Funding

This work was supported by a Research Grant number 2010/01385-5 from FAPESP (Sao Paulo Research Foundation, Brazil). P F S was the recipient of a CNPq (National Council for Scientific and Technological Development, Brazil) productivity grant number (PQ-302533/2011-7). R F A was the recipient of a CAPES (Coordination for the Improvement of Higher Education Personnel of the Ministry of Education in Brazil) fellowship (DR-2008-2012). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

#### Author contribution statement

P F S conceived and R F A, L G V and N Y carried out experiments, P F S and R F A analyzed data. P F S and R F A were involved in writing the paper. All authors had final approval of the submitted and published versions. All authors have read and agree with the manuscript as written.

#### Acknowledgements

The authors thank all the staff at the Unit of Translational Endocrine Pharmacology and Physiology at Laboratory of Pharmacology for technical support, and the agency GLMS Scientific Language Services for having improved the English in this manuscript.

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Received in final form 15 April 2015

Accepted 21 May 2015

Accepted Preprint published online 21 May 2015