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Adipocyte aminopeptidases in obesity and fasting

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ABSTRACT

This study checked the existence of a diverse array of aminopeptidase (AP) enzymes in high (HDM) and low (LDM) density microsomal and plasma membrane (MF) fractions from adipocytes of control, monosodium glutamate obese and food deprived rats. Gene expression was detected for ArgAP, AspAP, MetAP, and two AlaAP (APM and PSA). APM and PSA had the highest catalytic efficiency, whereas AspAP the highest affinity. Subcellular distribution of AP activities depended on metabolic status. Comparing catalytic levels, AspAP in HDM, LDM and MF was absent in obese and control under food deprivation; PSA in LDM was 3.5-times higher in obese than in normally fed control and control and obese under food deprivation; MetAP in MF was 4.5-times higher in obese than in food deprived obese. Data show new AP enzymes genetically expressed in subcellular compartments of adipocytes, three of them with altered catalytic levels that respond to whole-body energetic demands.

1. Introduction

Identifying the function of potential new enzymes that global genome sequencing efforts have revealed is one of the great challenges nowadays. Adipocytes are cells that depend on multiple enzymatic pathways to carry out their functions. The lack of a complete assembly of the genomic sequence and the presence of many predicted enzymes with unknown or unsure catalytic activities have hampered the complete view of the adipocyte's metabolic pathways (Rutkowski et al., 2015). Cys/Leu aminopeptidase (IRAP, insulin-regulated aminopeptidase, EC 3.4.11.3) in adipocytes is known to traffic between high and low density microsomal fractions toward the plasma membrane under stimulation by insulin (Keller et al., 1995, 2002 and Keller, 2004). The roles of IRAP in targeting AS160 protein to specialized vesicles containing GLUT4 and in regulating the retention of GLUT4 within the adipocytes were proposed based on data showing that the AS160 protein controls the amount of GLUT4 in plasma membrane and interacts with cytoplasmic domain of IRAP (Jordens et al., 2010). IRAP deficiency leads to suppression of plasminogen activator inhibitor type 1 expression in adipocytes and upregulation of uncoupling protein-1-mediated thermogenesis in brown adipose tissue and increased energy expenditure to prevent the development of obesity, and these facts suggest a therapeutic potential of IRAP/angiotensin (ANG) IV receptor blockade in diet-induced obesity (Niwa et al., 2015). IRAP has a preference for substrates containing the N-terminal Cys (Alponti et al., 2015), such as oxytocin. Indeed the obesity has been associated with reduced plasma oxytocin due to increased peptide degradation by liver and adipose tissue rather than changes in hormone synthesis (Gajdosechova et al., 2014). Dipeptidyl peptidase IV (DPPIV, EC 3.4.14.5) was recently identified in adipocytes from visceral and subcutaneous fat pads, being considered a new adipokine with local and systemic effects respectively in adipose tissue and bloodstream (Sell et al., 2013). Circulating levels of this aminopeptidase in obese patients are higher in insulin-resistant subjects than in insulin-sensitive ones, suggesting that DPPIV is like to be a marker of insulin resistance, independently of obesity (Sell et al., 2013). However, the functional role of DPPIV in adipose tissue remains to be elucidated. Besides these two aminopeptidase (AP) enzymes, there are no reports about other AP enzymes in adipocytes.

The relationship of AP enzymes with peptides involved in sensing the nutritional status and energy control balance is still poorly explored. Recently, the perspectives in this field were expanded by the demonstration that plasma activity levels of

Abbreviations: ANG, angiotensin; AP, aminopeptidase; APM, puromycin-insensitive neutral aminopeptidase; BSA, bovine serum albumin; DEPC, diethylpyrocarbonate; DPPIV, dipeptidyl peptidase IV; DTT, DL-dithiothreitol; EC, Enzyme Commission; ERAP, endoplasmic reticulum aminopeptidase; IRAP, insulin-regulated aminopeptidase; MSG, monosodium glutamate; polyQ, polyglutamine; PSA, puromycin-sensitive neutral aminopeptidase.

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puromycin-insensitive neutral AlaAP (APM, EC 3.4.112) are correlated with body mass, Lee index and retroperitoneal fat pad mass in food deprived rats (Alponti and Silveira, 2010). On the other hand, activity levels of diprotin A-insensitive DPPIV are correlated with body mass, Lee index and periepididymal adipose tissue mass and decreased 33% in monosodium glutamate (MSG) obese rats that are also food deprived (Alponti and Silveira, 2010). APM mapping in the hypothalamus and hippocampus (Alponti et al., 2011b), as well as of DPPIV/CD26 in the hypothalamus (Alponti et al., 2011c) showed the regulation of these two proteins in obese and food deprived rats. Many peptides involved in the intracellular machinery and/or in the regulation of energy homeostasis are substrates of AP enzymes such as bradykinin, polyglutamine (polyQ) sequences and ANG I, II and III (Martínez-Martos et al., 2011; Menzies et al., 2010; Prieto et al., 2003). The inhibitors of certain AP enzymes seem to be promising drug candidates to treat and prevent obesity-related diseases. Inhibition of DPPIV and APM and/or puromycin-sensitive neutral AlaAP (PSA, EC 3.4.11.14) has been preconized to suppress inflammatory immune responses (Reinhold et al., 2007). Gliptin class DPPIV inhibitors are effective to treat diabetes mellitus. Glucagon-like peptide-1 receptor agonists resistant to DPPIV hydrolysis are also effective to treat diabetes mellitus and have been associated with weight loss and reductions in systolic blood pressure (Russell, 2013). Basic AP (ArgAP, EC 3.4.11.6)/leukotriene A4 hydrolase inhibitor holds promise for improved anti-inflammatory properties (Stsiapanava et al., 2014). Acid AP (AspAP, EC 3.4.11.7) inhibitors that cross the blood–brain barrier have been believed to lead the way of a new class of antihypertensive agents (Marc et al., 2012). Furthermore, it is already known that fumagillin, a MetAP (EC 3.4.11.18) inhibitor, reduces the formation of adipose tissue in mice with diet-induced obesity (Lijnen et al., 2010). Beloranib, another MetAP inhibitor, is currently under phase 2a of clinical evaluation for the treatment of obesity (Joharapurkar et al., 2014).

The hypotheses of this study are that other aminopeptidases are also present in subcellular fractions of adipocytes and that obesity and food deprivation promote changes on the distribution of catalytic activities of these enzymes. In this sense, this study aimed to check the existence of genes and catalytic activities of APM, MetAP, ArgAP, AspAP and PSA, in addition to Leu/CysAP and DPPIV in high and low density microsomal and plasma membrane fractions from adipocytes of healthy control rats and whether these activities are altered in obese and food deprived statuses.

2. Material and methods

2.1. Animals and treatments

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) within a ventilated container (Alesco Ind. Com Ltda, Brazil) under 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 were subdivided into 2 groups, treated according to the methodology described by Alponti and Silveira (2010), as follow: i-obese group: received a daily subcutaneous (sc) bolus injection of L-glutamic acid monosodium salt (Sigma, USA) in saline 0.9% (4 mg/g body mass), in the cervical region, between 7:30–9:00 h of light period (13:30–15:00 h AM), at a maximum volume of 0.2 mL, until they were 10 days old; i-control group: received the same volume of saline 0.9% sc, in the same scheme as described above. On 22nd day the animals were weaned and the female was removed from the cage. On 90th day obesity was evaluated by Lee index, calculated by body mass $(g)^{0.33}$/naso-anal length (cm) (Kaufhold et al., 2002; Nakagawa et al., 2000). The i-obese group was then subdivided into 2 groups: obese (Lee index >0.300) and obese food deprived (obese animals fasted for 72 h). The i-control group was also subdivided into 2 groups: control (Lee index ≤0.300) and food deprived control (control animals fasted for 72 h). Food deprivation was performed by transferring pairs of animals into metabolic cages without food for 72 h. Except during this period, all experimental groups had access *ad libitum* to the same animal feed: Nuvilab® CR-11 (Sogorb, Brazil) with the following composition: 22% protein, 55% carbohydrate, 4% lipids, 9% fibers, 10% vitamins and minerals (total of 3 kcal/g). 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.

2.2. Adipocyte isolation

After euthanasia by decapitation, retroperitoneal fat pad was removed through the 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, Brazil) containing 25 mM HEPES (pH 7.5), 4% bovine serum albumin (BSA) (Sigma) and 45 mg of collagenase (Sigma), and then incubated at 37 °C, for 1 h, under gentle shaking. Subsequently, this mixture was washed with 8 volumes of buffered washing solution (115 mM NaCl, 0.8 mM $MgSO_4 \cdot 7H_2O$, 5.3 mM KCl, 1.4 mM $CaCl_2 \cdot 2H_2O$, 0.89 mM $NaH_2PO_4 \cdot H_2O$, 25 mM HEPES, 1 mM Na pyruvate, 145 mM BSA; pH 7.4, at 25 °C), and then filtered through a nylon mesh. This filtrate was centrifuged at 200 rpm for 1 min, at 25 °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 for 3 times more. The resultant suspension of isolated adipocytes was microscopically observed to verify the absence of vascular stroma.

2.3. Gene expressions

The RNA of adipocyte suspension from each animal was extracted using the sound RiboPure™ Kit (Applied Biosystems, USA) as recommended by the manufacturer and the final eluate containing RNA was obtained and stored at −20 °C until used.

The total RNA isolated was quantified and its purity was evaluated by Synergy™ H1 using the software Gen5™. The adequate quality of total RNA was checked by the existence of bands corresponding to 25S and 18S ribosomal RNA. As recommended by manufacturer, until 2 μg of total RNA in a maximum volume of 9 μL were used for reverse transcription procedure using the High Capacity RNA-to-cDNA kit (Applied Biosystems). The samples were then stored at −80 °C. The Taq man system® and the following probes (Life Technologies, Brazil) were used: ArgAP (Rn 00579477_m1, GenBank: NM_020216), AspAP (Rn 01400761_g1, GenBank: NM_001977), Leu/CysAP (Rn 00592103_m1, GenBank: NM_005575), DPPIV (Rn 00562910_m1, GenBank: NM_012789), MetAP (Rn 01509477_g1, GenBank: NM_022539), APM (Rn 00578763_m1, GenBank: NM_031012), PSA (Rn 01470353_m1, GenBank: NM_080395) and GAPDH (GenBank: NM_017008) (positive control). The thermal cycler conditions for the quantitative PCR (qPCR) reaction were: 1 cycle of 2 min at 50 °C, 1 cycle of 10 min