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APM/CD13 and FOS in the hypothalamus of monosodium glutamate obese and food deprived rats

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ABSTRACT

Protein (western blotting) and gene (PCR) expressions, catalytic activity of puromycin-insensitive membrane-bound neutral aminopeptidase (APM/CD13) and *in situ* regional distribution of CD13 and FOS immunoreactivity (ir) were evaluated in the hypothalamus of monosodium glutamate obese (MSG) and/or food deprived (FD) rats in order to investigate their possible interplay with metabolic functions. Variations in protein and gene expressions of CD13 relative to controls coincided in the hypothalamus of MSG and MSG-FD (decreased 2- to 17-fold). Compared with controls, the reduction of hypothalamic CD13 content reflected a negative balance in its regional distribution in the supraoptic, paraventricular, periventricular and arcuate nuclei. CD13-ir increased in the supraoptic nucleus in MSG (2.5-fold) and decreased in the paraventricular nucleus (2-fold) together with FOS-ir (1.5-fold) in FD. In MSG-FD, FOS-ir decreased (7-fold) in the paraventricular nucleus, while CD13-ir decreased in the periventricular (5.6-fold) and the arcuate (3.7-fold) nuclei. It was noteworthy that all these changes of CD13 were not related to catalytic activity of APM. Data suggested that hypothalamic CD13 plays a role in the regulation of energy metabolism not by means of APM enzyme activity.

1. 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 and protein hydrolysis [3] and the quantity of free amino acids is one of the limiting factors of proteolysis. The cellular activity measured by FOS immunoreactivity (ir) is known to be affected in the supramammilary and dorsomedial hypothalamic nuclei of obese mice and in the medial part of the perifornical hypothalamic nucleus (PHN) of food deprived mice [4]. Food deprived mice also have activation of the arcuate nucleus, but this effect is reversed after re-feeding [5]. Both lean and hyperlipidic diet-obese animals submitted to food deprivation for 24 h have an increased number of FOS-ir neurons in the arcuate nucleus (ArcNH), and a reduced number in the lateral hypothalamus (LH) and dorsomedial nucleus (DMH) [6]. Hyperlipidic diet-obese animals not submitted to food deprivation also showed changes in FOS-ir in hypothalamic areas such as LH, DMH and PHN [6]. A saturated fat diet also influenced FOS-ir in the hypothalamus, in a pattern consistent with its obesogenic effects [7]. Moreover, some peptides, including substance P, somatostatin, angiotensin III, vasopressin, kallidin, dynorphin, leu-and met-enkephalin and endorphin, exert significant effects on the nutritional status and regulation of energy balance by the central nervous system (CNS), and hydrolysis by exopeptidases such as neutral aminopeptidase, leading to inactivation or processing of these peptides, has been assumed to be a limitation for their biological activities [8–13]. In the CNS, the activity of membrane-bound neutral aminopeptidase is predominantly puromycin-insensitive (membrane alanine aminopeptidase, aminopeptidase M, APM-EC 3.4.11.2) [14,15] and has identity with a cluster of differentiation antigen 13 (CD13) [16]. Recent study suggested, through the analysis of correlation between enzyme activity of APM/CD13 and biometric parameters (naso-anal length and mass of retroperitoneal fat pad), the involvement of this enzyme in the regulation of somatostatin in the plasma of animals deprived of food for 72 h [17]. However, at our knowledge the APM/CD13 in the CNS has not yet been associated to obesity and food deprivation. To explore this association and its relationship with the cellular activity, the present work evaluated protein (western blotting) and gene expression (reverse transcription-polymerase chain reaction — RT-PCR) of CD13, regional distribution (immunohistochemistry) of CD13 and FOS and catalytic activity (hydrolysis of naphthylamide derivative substrate) of APM in the hypothalamus of monosodium glutamate (MSG) obese and/or food deprived (FD) rats.

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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), with food (commercially available ration; Nuvilab CR-1, Nuvital, Colombo, PR, Brazil; composed of 22% protein, 55% carbohydrate, 4% lipids, 8% fibers, 10% vitamins and minerals; total of 3 kcal/g) and tap water *ad libitum*, in a ventilated container (Alesco Ind Com, Monte Mor, SP, Brazil), with a controlled temperature (24 °C ± 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 AM and 9:00 AM of the 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 [18]. At 90 days, obesity was determined by the Lee index, calculated by body mass (in grams)$^{0.33}$/naso-anal length (in centimeters) [19,20]. These animals were then subdivided into 2 groups: MSG (obese = Lee index > 0.300) and MSG-FD (obese fasted for 72 h). Rats of the same age and strain, receiving 0.9% NaCl under the same experimental conditions, were subdivided into 2 groups: C (normal = Lee index ≤ 0.300 = control) and FD (normal fasted for 72 h). Food deprivation was performed by transferring pairs of animals, between 7:30 AM and 9:00 AM of the light period, into metabolic cages without food and with drinking water *ad libitum* for 72 h. Except during this period, all experimental groups had access *ad libitum* to the same ration of Nuvilab CR-1.

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).

2.2. Brain collection and fixation

The animals were anesthetized with a solution containing ketamine hydrochloride (König, Buenos Aires, BA, Argentina) (100 mg/mL) and xylazine chloridrate (Vetbrands, Paulínia, SP, Brazil) (100 mg/mL) by intraperitoneal injection (0.2 mL/100 g of body mass) between 4:00 AM and 6:30 AM during the light phase and subsequently received intracardiac bolus injection of 0.1 mL of sodium heparin solution (Roche, Rio de Janeiro, RJ, Brazil) (1000 IU/mL of saline). Cardiac perfusion was then performed with 0.9% NaCl plus 50 mmol/L phosphate buffer, pH 7.4, over a period of 5 to 10 min, at a flow rate of 12 to 15 mL/min, with a circulatory circuit opened by an incision in the right atrium to ensure the elimination of blood and to avoid the interference of its enzyme activities. The descending aorta was then clamped to ensure better perfusion of brain structures. The animals destined to immunohistochemistry studies were perfused for more 5 to 10 min under the same conditions but with a fixative solution composed of 4% paraformaldehyde (Synth, Diadema, Brazil) in 4% sodium borate (borax) (Synth, Diadema, Brazil), pH 9.5. After perfusion, the animals were decapitated with a guillotine; and the brains were removed for immediate use in immunohistochemistry, measurements of enzyme activity and protein content, RT-PCR and western blotting assays.

2.3. Preparation of solubilized membrane-bound fraction

After manual dissection, hypothalamic tissue (0.24–5.04 mm posterior to the bregma) [21] was homogenized in 10 mM Tris–HCl buffer (pH 7.4) (3 min, 800 rpm) (4 mL buffer/g wet wt) with a pestle mixer (Tecnal model Te-099, Piracicaba, Brazil) and ultracentrifuged (Hitachi model HIMAC CP60E, Tokyo, Japan) (100,000 × *g*, 35 min). The resulting supernatant was discarded and the resulting pellet was washed three times with the same buffer. The pellet was then homogenized (3 minutes, 800 rpm) in 10 mM Tris–HCl buffer, pH 7.4, plus 0.1% Triton X-100 (Calbiochem, San Diego, USA) and ultracentrifuged (100,000 × *g*, 35 min). The supernatant thus obtained was used to measure the protein content and western blotting. All steps were carried out at 4 °C.

2.4. Protein

Protein content was measured at 630 nm, in triplicates, using a BioRad protein assay kit (BioRad Laboratories, Hercules, USA) [22] in the Bio-Tek Power Wave X® spectrophotometer absorbance reader (Bio-Tek, Winooski, USA). Protein contents were extrapolated by comparison with the standard curve of bovine serum albumin (BSA) (Sigma, St. Louis, USA) in the same diluents as the samples.

2.5. Fluorometric quantification of catalytic activity of APM

The enzyme activity was quantified based on the amount of β-naphthylamine released [23] as the results of incubation (30 min, 37 °C), in 96-well flat bottom microplates, of 10 – 100 μL of solubilized membrane fraction of the hypothalamus, in triplicate, with pre-warmed solution of 0.125 mM L-Ala-β-naphthylamide (Sigma, St. Louis USA) (solubilized in 0.012 N HCl) in 0.05 M phosphate buffer, pH 7.4, containing 1 mM DL-dithiothreitol (Sigma, St. Louis, USA), 0.1 mg/mL BSA and 0.02 mM of puromycin (Sigma, St. Louis, USA). The naphthylamine content was estimated fluorometrically (with a FL600FA microplate fluorescence reader, Bio-Tek, Winooski, USA) at 460/40 nm emission wavelengths and 360/40 nm excitation wavelengths. The fluorescence value obtained at zero time (blank) was subtracted, and the relative fluorescence was then converted to picomoles of β-naphthylamine by comparison with the standard curve of β-naphthylamine (Sigma, St. Louis, USA) dissolved in the same diluents used in the incubation. APM activity was expressed as picomoles of hydrolyzed substrate per min (UP) per milligram of protein. The existence of a linear relationship between hydrolysis time and protein content in the fluorometric assay was a prerequisite. Considering enzyme activity measures as a comparative tool, the possible unspecific degradation during homogenization was not considered.

2.6. Western blotting of CD13

SDS-polyacrylamide gel eletrophoresis (SDS-PAGE) was performed with samples containing 100 μg protein/well, optimized for the separation of protein in the range of molecular weight of CD13 [24], in 10% separation gel and 4% stacking gel [25,26]. The patterns of molecular weight SDS-PAGE were used to analyze samples by extrapolation. Proteins were then transferred to a nitrocellulose membrane, for 1 h at 100 V. After being blocked for 1 h in a blocking buffer (0.2% Tween-20 in phosphate buffer 0.1 M, pH 7.2, plus BSA 5%), the membranes were incubated with rabbit anti-human CD13 polyclonal antibody (Santa Cruz Biotech., Santa Cruz, USA) (1:2,500) or rabbit anti-rat β-actin polyclonal antibody (Sigma, St. Louis, USA) (1:1,000) for 1 h. Subsequently, the membranes were washed with 0.2% Tween-20 in phosphate buffer 0.1 M, pH 7.2 and incubated with horseradish-peroxidase-conjugated goat anti-rabbit IgG (Amersham Pharmacia Biotech., Pittsburgh, USA) (1:5,000) for 1 h. The membranes were washed with the same buffer and then incubated with a chemiluminescence system (SuperSignal West Pico Trial Kit, Pierce Biotechnology, Rockford, USA), for 3 min at room temperature (20 °C) at an exposure time of 1 min. Densities of the bands were determined by GS 700 Densitometer® (Bio Rad Laboratories, Hercules, USA) using the image analysis software Molecular Analyst® (Bio Rad