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Oxidative stress programming in a rat model of postnatal early overnutrition — role of insulin resistance☆,☆☆

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Abstract

Postnatal early overfeeding (EO) is related to later development of overweight and other metabolic disorders. As oxidative stress is implicated in most human diseases, as obesity and diabetes, we decided to study some parameters related to oxidative stress and insulin signaling in liver from EO animals in adult life. To induce EO, litter size was reduced to three pups per litter (SL: small litter) and groups with normal litter size (NL:10 pups per litter) were used as control. After weaning, rats had free access to standard diet and water. Body weight and food intake were monitored daily and offspring were killed at 180 days-old. Significant differences had $P<.05$ or less. As expected, SL rats had hyperphagia, higher body weight and higher visceral fat mass at weaning and adulthood. In liver, postnatal EO programmed for lower catalase (−42%), superoxide dismutase (−45%) and glutathione peroxidase (−65%) activities. The evaluation of liver injury in adult SL group showed lower nitrite content (−10%), higher liver and plasma malondialdehyde content (+25% and 1.1-fold increase, respectively). No changes of total protein bound carbonyl or Cu/Zn superoxide dismutase protein expression in liver were detected between the groups. Regarding insulin signaling pathway in liver, SL offspring showed lower IRβ (−66%), IRS1 (−50%), phospho-IRS1 (−73%), PI3-K (−30%) and Akt1 (−58%). Indeed, morphological analysis showed that SL rats presented focal areas of inflammatory cell infiltrate and lipid drops in their cytoplasm characterizing a microsteatosis. Thus, we evidenced that postnatal EO can program the oxidative stress in liver, maybe contributing for impairment of the insulin signaling.

Keywords: Small litter; Reactive oxygen species; Insulin signaling

1. Introduction

In recent decades, the prevalence of childhood obesity has greatly increased worldwide [1]. It is known that nutritional, environmental and/or hormonal influences during critical periods early in life can permanently change the structure and function of body tissues and systems; this association is denominated metabolic programming [2], and it was confirmed by epidemiological and experimental data [3,4]. Studies in animal models have shown that excess of nutrition in perinatal life represents a risk factor for obesity and associated metabolic disturbances in adulthood [5–7]. Recently, in a systematic review and meta-analysis, Risnes et al. [8] showed a strong association between higher birth weight and increased risk of cancer deaths.

Rats raised in "small litters" (SL) are an established animal model to study short- and long-term consequences of childhood obesity [9]. This model of postnatal early overnutrition (EO) was associated with hyperphagia, obesity, hypertension and hyperinsulinemia in adult life [10–14]. Other studies have suggested that oxidative stress, the imbalance between cellular production of reactive oxygen species (ROS) and antioxidant defenses in cells, could be an early event in the development of obesity-related chronic diseases, such as cardiovascular diseases, diabetes mellitus and cancer [15,16]. Nutrient overload and obesity increase ROS generation and oxidative stress. Excessive nutrient in the metabolic pathways leads to an increased electron flux through mitochondrial electron transfer chain. The consequent electron leak from respiratory complex I and III of electron transfer chain leads to an increased production of ROS from the mitochondria, such as superoxide and hydrogen peroxide [15].

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Previously, we have shown in adult SL rats, the programming for overweight, higher total and visceral fat mass, lower high-density lipoprotein cholesterol, hyperphagia, central leptin resistance and thyroid hypofunction in adult life [6,7]. At weaning, SL rats have insulin resistance characterized by an increase in fasting glucose levels and hyperinsulinemia, while at 6 months old, these animals showed a slight impairment in glucose tolerance test, 60 and 120 minutes after glucose load, suggesting insulin resistance, despite basal normoglycemia and normoinsulinemia [7]. Other reports showed that older (8 months old) SL rats present insulin resistance, suggesting that insulin resistance in this experimental model seem to be age dependent [10,11].

Since ROS have been proposed as an unifying mechanism linking nutrient excess and obesity-associated disturbances, in the present study we evaluated some parameters related to oxidative stress in adult rats programmed by EO. In addition, considering that there are two-way association between excessive ROS and insulin resistance, we studied the insulin signaling in liver.

2. Methods and materials

The use of the animals according to our experimental design was approved by the Animal Care and Use Committee of the Biology Institute of the State University of Rio de Janeiro (CEUA/184/2007; CEUA/006/2009), which based their analysis on the principles adopted and promulgated by the Brazilian Law issued on November 8, 2008 [17,18]. Wistar rats were housed in a room with controlled temperature (25±1°C) and artificial dark–light cycles (lights on 07:00 h, lights off 19:00 h). Adult virgin female rats were caged with male rats (3:1) and after mating, each female was placed in an individual cage with free access to water and food until delivery.

2.1. Experimental model of postnatal EO

To induce EO during lactation, 3 days after birth, the litter size was adjusted to three male rats per litter (SL) [6,11]. Litter containing 10 pups per mother was used as control (NL). The rats analyzed were randomly chosen from 16 different litters (8 SL litters and 8 NL litters). After postnatal day 21 (PN21) that corresponds to weaning period, both groups have free access to water and standard diet. During lactation, body weight (BW) gain was daily monitored and from weaning until PN180, body weight and food intake (g/100g BW) were monitored every 4 days.

At PN180, rats were killed after to be anaesthetized with pentobarbital (0.06 g/kg BW) in order to collect blood, liver and visceral fat mass (VFM). The blood was collected by cardiac puncture and poured in a tube containing EDTA. The VFM (mesenteric, epididymal and retroperitoneal white adipose tissue) was excised and immediately weighed for evaluation of central adiposity. Plasma and liver samples were frozen at −80°C until analysis.

2.2. Determination of antioxidant enzyme activities in liver

Liver samples of 200 mg were homogenized in potassium phosphate buffer with EDTA in mechanical homogenizer (CT-136 model from Cientec–laboratory equipment, Campinas, SP, Brazil). After centrifugation, homogenates were stored at −80°C until analysis. The total protein content was determined by the Bradford method [19].

Total superoxide dismutase (SOD) activity was assayed by measuring the inhibition of adrenaline auto-oxidation as absorbance at 480 nm [20]. Catalase (CAT) activity was measured by the rate of decrease in H_2O_2 at 240 nm according to the method of Aebi [21]. Glutathione peroxidase (GPx) activity was evaluated according to Flohé & Günzler [22] by measuring the oxidation of NADPH at 340 nm in the presence of H_2O_2.

2.3. Nitrite assay

The yield of radical nitric oxide (NO) an indirect measurement of nitric oxide content was evaluated by Griss reaction through quantification of nitrite (NO_2^-) in liver at 540 nm [23].

2.4. Thiobarbituric acid reactive substances (TBARS)

Lipid peroxidation was measured by malondialdehyde (MDA) concentration using the TBARS method as previously described [24,25]. Briefly, plasma and liver homogenates were mixed with 1 ml of 10% trichloroacetic acid and 1 ml of 0.67% thiobarbituric acid (Sigma Chemical, St. Louis, MO, USA); subsequently they were heated in a boiling water bath for 30 min. The absorbance of the organic phase containing the pink chromogen was measured spectrophotometrically at 532 nm. MDA equivalents were expressed in nMol/mg protein.

2.5. Protein oxidation

Protein oxidation was evaluated in liver accordingly Levine et al. [26] as carbonyl groups reacting with 2,4-dinitrophenyl-hydrazine (Sigma). Values of absorbance were obtained at 380 nm and expressed in nmol of carbonyl by 0.5 mg of protein.

2.6. Western blotting analysis

Liver samples were homogenized in cold lysis buffer (50 mM Hepes, pH 6.4, 1 mM MgCl2, 10 mM EDTA and 1% Triton X-100) containing protease inhibitors (10 μg/μl aprotinin, 10 μg/μl leupeptin, 2 μg/μl pepstatin and 1 mM phenylmethylsuphonic fluoride, Sigma-Aldrich, St. Louis, MO, USA) using a Ultra-Turrax homogenizer (IKA Werke, Staufen, Germany). After centrifugation, homogenates were stored at −20°C. The total protein content was determined by the BCA protein assay kit (Pierce, Rockford, IL, USA).

Samples (30 μg total protein) were electrophoresed in 10–12% Tris-glycine sodium dodecyl sulfate polyacrylamide gels. Proteins were transferred for polyvinylidene fluoride membranes (Hybond ECL; Amersham Pharmacia Biotech, London, UK), blocked in 5% dry milk in Tween-20 tris buffered saline (T-TBS; 0.02 M Tris/0.15 M NaCl, pH 7.5 containing 0.1% Tween 20) at room temperature for 1 h, washed 3× with T-TBS and incubated with the primary antibodies (Cu/Zn SOD, IR β, IRS1, phospho-IRS1, PI3-K, Akt1 and phospho-Akt1 at 1:500 concentration) overnight at 4°C. Primary antibodies were purchased from Santa Cruz Biotechnology (San Francisco, CA, USA). After washing 3× with T-TBS, blots were incubated with appropriate secondary antibodies at 1:5000 concentration (Santa Cruz Biotechnology) for 1 h and then incubated with streptavidin (Zymed, Carlsbad, CA, USA) in the same dilution of the secondary antibody for 1 h. Blots were developed with diaminobenzamidine (DAB; Sigma Chemical) as chromogenic substrate or with enhanced chemiluminescence (ECL; Amersham Biosciences, Piscataway, NJ, USA).

2.7. Liver histology

Liver samples were fixed in formalin (freshly prepared 1.27 mol/L formaldehyde, 0.1 M phosphate-buffered saline, pH 7.2) and embedded in paraffin to non-serial sections of 5 μm. Sections were placed in glass slides to stain in hematoxylin/eosin. The morphological study was performed utilizing digital images, acquired at random (TIFF format, 36-bit color, 1360x1024 pixels) with an Olympus DP71 camera and an Olympus BX40 epifluorescence microscope (Olympus, Tokyo, Japan).

2.8. Statistical analysis

Data are reported as mean±S.E.M. The *GraphPad Prism 4* program (GraphPad softwares, La Jolla, CA, USA) was used for statistical analyses and graphics. Two-way analysis of variance and Bonferroni post test were used to analyze body weight and food intake changes. Cu/Zn SOD expression and insulin signaling were analyzed by the non-parametric Mann–Whitney *U* test. The other experimental observations were analyzed by unpaired Student's *t* test, with significance level set at $P<.05$.

3. Results

3.1. Body weight, food intake and visceral fat mass

Body weight and food intake from weaning (PN21) to the sacrifice (PN180) are shown in Fig. 1. Offspring overfed during lactation (SL) had higher body weight than NL rats from PN7 until the end of lactation (+10%, $P<.0001$, Fig. 1A). SL rats remained overweight until PN180 (+15%, $P<.0005$, Fig. 1B). SL group presented a higher relative food intake from weaning until adulthood (PN180: +7%, $P<.05$, Fig. 1C). Also VFM was higher (+92%, $P<.0001$, Fig. 1D) in SL rats compared to NL rats.

3.2. Evaluation of oxidative stress parameters

As shown in Fig. 2, adult SL offspring showed lower CAT (−42%, $P<.0001$; Fig. 2A), SOD (−45%, $P<.0001$, Fig. 2B) and GPx activities (−65%, $P<.0001$, Fig. 2C) than the NL group. Despite the lower SOD activity, Western blot analysis showed that Cu/Zn SOD content was not different between the groups (NL:101.49±6.36 vs. SL:86.60±5.43).

As depicted in Fig. 3, liver nitrite bioavailability was lower in SL than NL group (−10%, $P<.0001$, Fig. 3A). Oxidative damage