Food and Chemical Toxicology 92 (2016) 245–255

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

High calcium diet improves the liver oxidative stress and microsteatosis in adult obese rats that were overfed during lactation

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ARTICLE INFO

Article history:
Received 27 January 2016
Received in revised form
24 March 2016
Accepted 17 April 2016
Available online 21 April 2016

Key words:
Overnutrition
High calcium diet
Oxidative stress
Liver dysfunction

ABSTRACT

Obesity is related to diabetes, higher oxidative stress and nonalcoholic fatty liver disease, and dietetic therapies, for instance calcium-rich diet, can improve these dysfunctions. Rats raised in small litters (SL) had increased fat depots and insulin resistance at adulthood associated with higher liver oxidative stress and microsteatosis. Thus, we evaluated if dietary calcium can improve these changes. In PN3, litter size was adjusted to 3 pups (SL group) to induce overfeeding, while controls had 10 pups until weaning. At PN120, SL group was randomly divided into: rats fed with standard chow or fed with calcium supplementation (SL–Ca group, 10 g/kg chow) for 60 days. At PN180, dietary calcium normalized food consumption, visceral fat, plasma aspartate aminotransferase (AST) and glycaemia. Concerning oxidative balance, calcium restored both higher hepatic lipid peroxidation and protein carbonylation as well as higher plasma lipid peroxidation. Higher fatty acid synthase (FAS) content, steatosis and lower protein kinase B (Akt) in SL group were normalized by dietary calcium and SL–Ca rats had lower hepatic cholesterol. Thus, calcium supplementation improved the insulin sensitivity, redox balance and steatosis in the liver. Therefore, dietary calcium can be a promising therapy for liver disease in the metabolic syndrome.

1. Introduction

Westernized lifestyle causes high rates of overweight and obesity in the population (Barnes, 2015) as well as their related comorbidities, such as type 2 diabetes, hypertension, dyslipidemia, and nonalcoholic fatty liver disease (Grundy, 2015). Concerning to health, economic and social repercussion of obesity, several anti-obesogenic therapies have been proposed lately (Maksimov et al., 2015). Among these efforts, the use of high calcium diet is considered as an important dietetic strategy against the obesity and overweight (Chaplin et al., 2015; Nobre et al., 2011, 2012; Yoda et al., 2015). Previously our group demonstrated that dietary calcium supplementation during 60 days in an experimental obesity model (early weaning) was able to reduce the hyperphagia by correcting the leptin signaling in the hypothalamus (Nobre et al., 2012) and glucagon-like peptide 1 (GLP1) response (Quitete et al., 2015). Hence, these changes probably caused a reduction of body weight and visceral adiposity in this model.

In obesity, the high caloric diet intake is related to the development of the oxidative stress by the high production of reactive oxygen and nitrogen species (ROS and RNS, respectively) and decreased neutralization capacity (Maurya et al., 2015). The ROS/RNS are highly instable, and react with the surround molecules, causing lipid peroxidation, protein carbonylation and DNA damage (Apel and Hirt, 2004). In supra-physiologic level, these modifications cause the loss of molecular function as well as lead to several diseases and aging. Among the diseases, it has been show that the oxidative stress has an important contribution to the development of liver steatosis (Polimeni et al., 2015).

Steatosis is an important repercussion of obesity, especially in liver, since this organ has a crucial role in glucose level management and lipid distribution after a meal and during fasting (Milić

Abbreviations: 4-HNE, 4-hydroxynonenal; Akt, protein kinase B; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CAT, catalase; FAS, fatty acid synthase; GLP1, glucagon-like peptide 1; GPx, glutathione peroxidase; MDA, malondialdehyde; NADPH oxidase, nicotinamide adenine dinucleotide phosphate-oxidase; RNS, reactive nitrogen species; ROS, reactive oxygen species; SL, small litters; SL–Ca, SL fed with calcium supplementation; SOD, superoxide dismutase.

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http://dx.doi.org/10.1016/j.fct.2016.04.015
0278-6915/

et al., 2014). The obesity increases lipid delivery to the liver from the diet and from the adipose tissue and increases liver *de novo* lipogenesis, due to the higher plasma glucose (Donnelly et al., 2005). The pro-oxidative condition in the liver causes oxidative damage, impairing the liver metabolic function, for instance insulin sensitivity (Lomonaco et al., 2012; Conceição et al., 2013). Recently, Chaplin et al. (2015) demonstrated that a high fat diet with high calcium content (12 g/kg) prevented the lipid accumulation in the liver. These authors did not report any oxidative parameter. Based on this finding, it remains unclear if dietary calcium is able to protect against liver oxidative stress in obesity models.

In the current study, we used rats raised in 'small litters' (SL) to induce postnatal overnutrition, which is a well-established animal model to evaluate short and long-lasting consequences of childhood obesity. We already showed that the reduction of litter size was associated with increased plasma and liver oxidative stress as well as higher triglyceride content in the liver at weaning (Conceição et al., 2015a) and adulthood (Conceição et al., 2013). More recently, we demonstrated that calcium supplementation is able to reduce the food intake of adult SL rats through normalization of higher NPY found in SL untreated group (Conceição et al., 2016). Therefore, using tissues (plasma and liver) from the previous published study (Conceição et al., 2016), our main objective was to evaluate whether dietary calcium supplementation during 60 days, from PN120 to PN180, is capable to prevent the development of oxidative stress and microsteatosis in the liver of obese SL rats, improving these metabolic disturbances.

2. Materials and methods

2.1. Reagents

Calcium carbonate, formaldehyde, ethanol, xylol, paraffin, trichloroacetic acid, RIPA buffer reagents, and hydrogen peroxide were purchased from Vetec Quimica Fina Ltda (Duque de Caxias, Brazil). Insulin radioimmunoassay kit was purchased from ICN Pharmaceuticals, Inc. (Orangeburg, USA). Triglyceride and cholesterol kits were purchased from Biosystem (Paraná, Brazil) and from Bioclin (Minas Gerais, Brazil). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) kits were purchased from Doles Reagentes (Goiás, Brazil). Primary and secondary antibodies were purchased from Abcam (Cambridge, UK), Santa Cruz Biotechnology (Finnell St, USA), Invitrogen Corporation (California, USA), and Sigma–Aldrich (St. Louis, EUA). Protease inhibitor cocktail was purchased from F. Hoffmann-La Roche Ltd. (Basel, CH). BCA protein assay kit was purchased from Thermo Scientific (California, USA). Nitrocellulose membranes and chemiluminescence ECL plus were purchased from Amersham Pharmacia Biotech (London, UK). Thiobarbituric acid and 1,1,3,3-tetrametoxipropane were purchased from Milipore (Billerica, EUA). 2,4-dinitrophenyl-hydrazine, catalase bovine, β-Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate, glutathione reductase from baker's yeast (*S. cerevisiae*), and glycine were purchased from Sigma–Aldrich.

2.2. Animals

The experimental design was previously approved by the Animal Care and Use Committee of the Biology Institute of the State University of Rio de Janeiro. Wistar rats were housed under controlled temperature room (23 ± 3 °C), light (12 h light/dark cycle), and had free access to food and water.

For mating, adult twenty nulliparous female Wistar rats (*Rattus norvegicus*) were placed with ten adult male rats in a 2 to 1 ratio during 5 days. The pregnant females were housed in individual cages until the delivery. After birth, all litters were adjusted to 10 male pups per dam. The female pups were replaced by male pups from other litters.

At third day of life, to induce early overfeeding, the litter size was reduced to three male pups per dam (SL group, n = 10). The control group (NL group, n = 10) was kept with ten pups per dam until the weaning period (Conceição et al., 2015a, b). Only male pups were studied because it was already published that there are no gender differences at weaning in the SL model especially concerning the body weight, fat mass, lean mass, leptinemia, energy expenditure and respiratory exchange ratio (Stefanidis and Spencer, 2012).

2.3. High calcium diet during two months

In order to evaluate if this micronutrient can decrease the greater visceral adiposity in SL rats, the calcium supplementation was administered during two months. This chow was done in our laboratory. For this, the standard rat chow bran was supplemented with calcium carbonate (10 g of $CaCO_3$/kg chow), and mixed with an industrial food mixer, followed by water addiction. Then the chow were pelletized, and dried in an industrial heater (60 °C) during 12 h. This calcium concentration is twice the amount recommended to rodents (5 g/kg of chow) (Reeves, 1997). Our group already reported that this concentration did not alter diet palatability, since lean control rats fed with this diet did not change the food intake, body weight, fat depot or leptin level (Nobre et al., 2011; Nobre et al., 2012).

From PN120 until PN180, SL animals were subdivided into two groups: 1) SL − received standard chow (n = 10); 2) SL–Ca − received standard chow supplemented with calcium carbonate (n = 10). The NL group (n = 10) received standard rat chow.

2.4. Insulin evaluation

Blood samples were centrifuged (1500 × g/20 min per 4 °C) in heparinized tubes to obtain plasma, which was kept at − 80 °C until assay. Plasma insulin was determined using a radioimmunoassay kit with an assay sensitivity of 0.1 ng/ml and an intra-assay variation of 4.1%. The measurement was performed in one assay and samples were analyzed in duplicate.

2.5. Lipid determination

Plasma triglycerides and total cholesterol were analyzed using commercial test kits.

Hepatic triglyceride and cholesterol contents were measured by colorimetric assay using by commercial kit at 500 nm (Hidex, Turku, Finland), after total liver lipid content extraction with isopropanol (Folch et al., 1957).

2.6. Plasma transaminases measurement

The plasma activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were quantified by Reitman and Frankel method (1957) using colorimetric kits. The results were expressed as un.F.R./mL.

2.7. Hepatic morphological analysis

Liver samples were fixed in formaldehyde 0.1 M phosphate-buffered saline (pH 7.2), and dehydrated, cleared, and paraffin-embedded. Nonconsecutive slices of 10 μm sections thick were obtained and stained with hematoxylin/eosin. From each rat, 3-blinded laminas were obtained, and from each lamina were taken