Experimental Gerontology 51 (2014) 54–64

Contents lists available at ScienceDirect

Experimental Gerontology

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

Food restriction enhances oxidative status in aging rats with neuroprotective effects on myenteric neuron populations in the proximal colon

João Paulo Ferreira Schoffen [a,*], Ana Paula Santi Rampazzo [b], Carla Possani Cirilo [b], Mariana Cristina Umada Zapater [b], Fernando Augusto Vicentini [b], Jurandir Fernando Comar [c], Adelar Bracht [c], Maria Raquel Marçal Natali [b]

[a] Center of Biological Sciences, State University of the North of Paraná (UENP), Bandeirantes, Paraná, Brazil
[b] Department of Morphological Sciences, State University of Maringá (UEM), Maringá, Paraná, Brazil
[c] Department of Biochemistry, State University of Maringá (UEM), Maringá, Paraná, Brazil

ARTICLE INFO

Article history:
Received 10 August 2013
Received in revised form 3 January 2014
Accepted 7 January 2014
Available online 14 January 2014

Section Editor: Christian Humpel

Keywords:
Food restriction
Aging
Oxidative stress
Myenteric plexus
Muscle layer

ABSTRACT

Food restriction may slow the aging process by increasing the levels of antioxidant defenses and reducing cell death. We evaluated the effects of food restriction on oxidative and nutritional status, myenteric cell populations, and the colonic muscle layer in aging rats. Wistar rats were distributed into control groups (7, 12, and 23 months of age) and subjected to food restriction (50% of normal diet) beginning at 7 months of age. The animals were sacrificed, and blood was collected to evaluate its components and markers of oxidative status, including thiobarbituric acid-reactive substances, reduced glutathione, catalase, glutathione peroxidase, and total antioxidant capacity. The proximal colon was collected to evaluate HuC/D and neuronal nitric oxide synthase (nNOS)-positive and -negative myenteric neurons, S-100 glial cells, and the muscle layer. Age negatively affected oxidative status in the animals, which also increased the levels of total cholesterol, protein, and globulins and increased the thickness of the muscle layer. Aging also reduced the number and hypertrophied glial cell bodies, HuC/D neurons, and nNOS-negative and -positive neurons. An improvement was observed in oxidative status and the levels of total cholesterol and triglycerides with food restriction, which also provided neuroprotection of the intrinsic innervation. However, food restriction accentuated the loss of enteric glia and caused hypertrophy in the muscle layer at 23 months. Food restriction improved oxidative and nutritional status in rats and protected HuC/D neurons and nNOS-negative and -positive neurons against neuronal loss. Nevertheless, food restriction caused morphoquantitative changes in glial cell populations, with possible interference with colonic neuromuscular control.

1. Introduction

Aging is a complex process that involves progressive and deleterious changes in cellular function, partially resulting from the increased production of reactive oxygen species (ROS), senescence, cellular dysfunction, or cell death (Finkel and Holbrook, 2000; Oliveira and Schoffen, 2010). Oxidative and metabolic changes that occur during aging increase the vulnerability of cells and organs to several diseases (Friedlander, 2003; Mattson and Magnus, 2006).

Most studies in animals and humans have reported a quantitative decrease in the number of neurons in the enteric nervous system (ENS) with aging (Bernard et al., 2009; El-Salhy et al., 1999; Phillips et al., 2003; Saffrey, 2013), but not in all studies (Gamage et al., 2013; Peck et al., 2009). Changes in the structure or quantity of enteric neurons may explain disorders of the digestive system (Bernard et al., 2009; Wade, 2002). Common changes include intrinsic innervation and alterations in smooth muscle (i.e., target tissue responsible for neuronal development, maintenance, and plasticity) that subsequently alter gastrointestinal motility and colonic transit time, which is often slowed in elderly people (Madsen and Graff, 2004; Wiskur and Meerveld, 2010). Thus, chronic constipation affects up to 27% of the elderly population (Bouras and Tangalos, 2009).

Variations in the density of different myenteric neuronal populations in the gastrointestinal tract (GIT) during aging have been reported (Belai et al., 1995; Gagliardo et al., 2008; Phillips et al., 2003; Porto et al., 2012), indicating differences in the susceptibility of certain types of neurons according to the subpopulation and segment analyzed. Notably, the colon is one region of the GIT that is more vulnerable to aging (Phillips and Powley, 2001, 2007). With regard to the enteric glial cell population, changes in the number and properties of these cells as age advances have been poorly investigated (Cirilo et al., 2013; Phillips et al., 2004).

* Corresponding author at: Center of Biological Sciences, State University of the North of Paraná (UENP), Rodovia BR-369 Km 54, Vila Maria-Bandeirantes CEP: 86360-000, Paraná, Brazil. Tel.: +55 43 3542 8042; fax: +55 43 3542 8008.
E-mail address: jpschoffen@uenp.edu.br (J.P.F. Schoffen).

0531-5565/$ – see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.exger.2014.01.001

Neuronal loss may be attributable to several causes, including the increased production and accumulation of free radicals (Thrasivoulou et al., 2006), the reduction of neurotrophic factors derived from glial cells (Wade, 2002), the activation of apoptosis, changes in gene expression or mutations in certain genes (Wiley, 2002), changes in Ca^{2+} signaling (Mattson, 2007), and mitochondrial dysfunction (Wade, 2002).

Studies have indicated that a reduction of caloric intake may slow aging by reducing the incidence of age-related chronic diseases (e.g., diabetes, cancer, cardiovascular disease, and neurodegenerative disease), extending the lifespan of various species, including mammals (Bordone and Guarente, 2006; Sohal and Weindruch, 1996).

Food restriction, also referred to as caloric restriction, may also reduce the rate of tumor formation and increase the efficiency of the immune system (Wachsman, 1996), slow metabolism, reduce apoptosis (Zhang and Herman, 2002), enhance neurotrophic signaling (Thrasivoulou et al., 2006), increase the plasticity, longevity, and survival of neurons (Cowen et al., 2000), and minimize the effects of oxidative stress generated during cellular respiration by reducing the levels of free radicals (Barja, 2002).

The literature indicates that myenteric neuron neurodegeneration begins in adulthood and continues through middle age and old age, with different times of degeneration among intestinal segments (Phillips and Powley, 2001, 2007). However, few studies have investigated the possible beneficial effects of food restriction on the behavior of myenteric intrinsic innervation in aging (Cirilo et al., 2013; Cowen et al., 2000; Porto et al., 2012; Thrasivoulou et al., 2006), and fewer studies have correlated this behavior with oxidative status (Cirilo et al., 2013; Thrasivoulou et al., 2006). No studies of which we are aware of have morphoquantitatively investigated glial and myenteric neurons in the large intestine in animals subjected to prolonged food restriction.

Given the possible occurrence of alterations in the myenteric plexus during the aging process and beneficial effects of reduced food intake, we evaluated the effects of food restriction on morphoquantitative aspects of the overall neuronal population (HuC/D-positive), nitrergic neuron subpopulation (nNOS-positive), cholinergic neuron subpopulation (nNOS-negative), and glial cell population (S-100) in the myenteric ganglia and muscle layer of the proximal colon and examined nutritional and oxidative status in rats during the aging process. We sought to provide data that may minimize the deleterious effects of aging.

2. Material and methods

2.1. Animals and treatment

Thirty male Wistar rats (*Rattus novergicus*; 7, 12, and 23 months of age) were used. They were kept in polyethylene boxes on a 12 h/12 h light/dark cycle under controlled temperature (22 ± 2 °C). The animals were assigned to five groups: control groups (7 months of age [C7 group], 12 months of age [C12 group], and 23 months of age [C23 group]) and experimental groups (food restriction from 7 to 12 months of age [RA12 group] and food restriction from 7 to 23 months of age [RA23 group]).

The control groups were fed *ad libitum* with standard NUVILAB-NUVITAL rodent chow. The amount of food consumed was controlled by supplying 100g daily for each animal and weighing the remainder, thus obtaining daily intake. In the experimental groups beginning at 7 months of age, the animals received half of the average daily intake of the rats fed *ad libitum* for 5 and 16 months (RA12 and RA23 groups, respectively). The body weights of the animals were recorded every 2 weeks, and food intake was monitored every month.

2.2. Tissue collection

At 7, 12, and 23 months of age, after fasting for 10 h, the rats were weighed and anesthetized with intraperitoneal sodium thiopental (Thionembutal; 40 mg/kg of body weight). The naso-anal distance was measured to determine the Lee index (body weight$^{1/3}$ [g]/naso-anal distance [cm] × 1000) (Bernardis and Patterson, 1968).

Blood was collected by cardiac puncture to measure biochemical parameters, including total protein, albumin, globulin, glucose, triglycerides, total cholesterol, aspartate aminotransferase (AST), and alanine aminotransferase (ALT), and analyze oxidative status in the animals.

Laparotomy was then performed to remove and weigh retroperitoneal and periepididymal adipose tissues. The large intestine was collected, and the length was measured. Samples of the proximal colon (characterized by the end of the ileum–cecum–colic ampoule until the disappearance of mucosal oblique folds) underwent immunohistochemical double staining with HuC/D (i.e., a pan-neuronal marker) and nNOS, double staining with HuC/D and S-100 (i.e., a marker of glial cells), and histological processing to morphometrically analyze the intestinal muscle layer.

All of the procedures were approved by the Ethics Committee for Animal Experimentation of the State University of Maringá, Brazil.

2.3. Biochemical analysis of blood components

To analyze total protein, albumin, globulin, triglycerides, and total cholesterol, blood was collected and placed in test tubes to obtain serum. For the glucose assay and determination of the activity of AST and ALT, blood sample was added to test tubes that contained 3 mmol/L fluoride ethylenediaminetetraacetic acid (EDTA). Both samples were centrifuged at 3000 rotations per minute for 15 min to obtain plasma using an Analisa kit (Gold Analisa Diagnóstica, Minas Gerais, Brazil).

2.4. Evaluation of oxidative status

2.4.1. Blood preparation

The collected blood was placed in tubes that contained 3 mmol/L EDTA as an anticoagulant. The blood was then centrifuged at 1000 ×*g* for 10 min, and the supernatant (plasma) was separated and frozen in liquid nitrogen for the analysis of lipid peroxidation and total antioxidant capacity. The residual sediment that contained erythrocytes was subjected to two cycles of washes by resuspension and centrifugation at 1000 ×*g* with 0.9% NaCl. The erythrocyte suspension was hemolyzed with 10 volumes of chilled deionized water and centrifuged at 4000 ×*g* for 10 min. The supernatant was used to determine reduced glutathione and the activity of catalase and glutathione peroxidase. All of the procedures were conducted at temperatures below 4 °C.

2.4.2. Analytical procedures for assessing oxidative stress

The levels of lipid peroxidation were determined by the thiobarbituric acid-reactive substance (TBARS) method as described by Buege and Aust (1978) to evaluate oxidative injury. The total antioxidant capacity (TAC) of the plasma was determined by the colorimetric method using 2,2′-azinobis (3-etylbenzthiazoline-6-sulphonic acid) (ABTS) as described by Erel (2004). The levels of reduced glutathione (GSH) in erythrocytes were determined by spectrofluorimetry as described by Hissin and Hilf (1976). Catalase activity in erythrocytes was assessed by the enzymatic decomposition of H_2O_2 measured by spectrophotometry at 240 nm as described by Aebi (1974). The activity of glutathione peroxidase in erythrocytes was determined by the decrease in absorbance caused by the decomposition of NADPH-dependent H_2O_2 at 340 nm at 25 °C (Paglia and Valentine, 1967; Tappel, 1978).

2.5. Immunohistochemistry of myenteric neurons and glia

Samples of the proximal colon were washed with 0.1 M phosphate-buffered saline (PBS; pH 7.4) to remove residues, and its ends were tied off, filled and distended with Zamboni's fixative, and immersed in the same fixative for 18 h at 4 °C. The samples were then opened and