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Supplementation with L-glutathione improves oxidative status and reduces protein nitration in myenteric neurons in the jejunum in diabetic *Rattus norvegicus*

Catchia Hermes-Uliana[a,*], Flávia Cristina Vieira Frez[b], Camila Caviquioli Sehaber[b], Francielle Veiga Ramalho[b], Fernando Pinheiro de Souza Neto[c], Rubens Cecchini[c], Flávia Alessandra Guarnier[c], Jacqueline Nelisis Zanoni[b]

[a] Universidade Federal de Mato Grosso do Sul, 79613-000 Três Lagoas, MS, Brazil
[b] Departamento de Ciências Morfológicas, Universidade Estadual de Maringá, 08020-900 Maringá, PR, Brazil
[c] Departamento de Ciências Patológicas, Universidade Estadual de Londrina, 86051-980 Londrina, PR, Brazil

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ABSTRACT

Diabetes mellitus is a syndrome with multiple etiologies, characterized by chronic hyperglycemia that increases the production of reactive oxygen species and decreases antioxidant defenses. The present study evaluated oxidative stress parameters and protein nitration in myenteric neurons in the jejunum in diabetic rats supplemented with L-glutathione. Rats (90 days of age) were distributed into four groups ($n = 6$/group): normoglycemic (N), normoglycemic supplemented with L-glutathione (NGT), diabetic (D), and diabetic supplemented with L-glutathione (DGT). At 210 days of age, the animals were sacrificed, and the jejunum was collected, washed, and subjected to various procedures: *tert*-butyl hydroperoxide chemiluminescence (CL), determination of total antioxidant capacity (TAC), determination of catalase activity, quantification of nitric oxide (NO), and double-labeling of HuC/D-immunoreactive myenteric neurons and nitrotyrosine (3-NT). Diabetes increased oxidative stress in the jejunum in the D group, reflected by increases in lipid peroxidation, TAC, catalase activity, and NO. The D group exhibited an increase in the percentage of myenteric neurons that were double-labeled with 3-NT. Supplementation with L-glutathione did not cause differences in the average CL curves between the D and DGT groups, but reductions of TAC and catalase activity were observed. Supplementation with L-glutathione promoted a reduction of neurons that contained 3-NT in the DGT group. Diabetes mellitus promoted oxidative stress in the jejunum, and supplementation with L-glutathione improved oxidative status by preventing protein nitration in myenteric neurons in diabetic animals that received supplementation.

1. Introduction

Diabetes mellitus (DM) is a metabolic disorder with multiple etiologies and characterized by chronic hyperglycemia (Conget, 2002). It affects approximately 7% of the population, and 50% of people are unaware of their condition. An estimated 552 million people will be afflicted by DM by 2030 (Whiting et al., 2011), with a new case every 5 s (American Diabetes Association, 2012; Takaku et al., 2006). Diabetes can lead to such complications as retinopathy, nephropathy, neuropathy, and endocrine dysfunction. The role of oxidative/nitrosative stress in these complications has been the subject of much interest (Reis et al., 2008). Numerous studies have shown that oxidative stress is incrementally present during the course of DM and significantly contributes to its progression (Chandrasekharan et al., 2011; Vincent et al., 2004).

The relationship between oxidative stress and tissue damage in diabetic rats has been evaluated in the stomach (Dias et al., 2004; Kochar and Umathe, 2009), the liver (Dias et al., 2004), the latissimus dorsi muscle (De Angelis et al., 2000), erythrocytes (Yadav et al., 1996), the kidneys (Kedziora-Kornatowska et al., 2002), and the brain (Kosenko et al., 1999). In line with this, oxidative/nitrosative stress has also demonstrated to be related to the pathogenesis of the famous neurodegeneration associated with diabetes (Liu et al., 2014; Stavniichuk et al., 2014). Gut is an organ full of nervous tissue, and also know as the second brain (Furness, 2012). Few studies have analyzed the effects of DM on the gut (Kochar and Umathe, 2009), and less is known about oxidative stress in myenteric neurons.

Decreases in neurons in the myenteric plexus were detected in

* Corresponding author at: Universidade Federal de Mato Grosso do Sul UNID. II: Av. Ranulpho Marques Leal, 3484/Cx, Postal n 210, 79613-000 Três Lagoras, MS, Brazil.
E-mail address: catchiahermes@gmail.com (C. Hermes-Uliana).

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various intestinal segments in diabetic rats (Zanoni et al., 2003). Nitrergic inhibitory neurons play a crucial role in controlling the motility of the gastrointestinal tract (Jarvinen et al., 1999). Nitric oxide (NO) is lipophilic and crosses the membranes of effector cells to produce its physiological effects. However, it may also behave as a free radical from nitrogen or react directly with proteins, thus causing nitrotyrosine formation, an irreversible reaction that alters protein conformation and, consequently, action. In addition, other free radicals that are generated endogenously can interact with NO· (Halliwell and Gutteridge, 2007). Some studies have demonstrated an increase in nitrotyrosine in myenteric neurons, or an increase in the production of free radicals, during the induction of inflammatory conditions, (Rivera et al., 2011; Van Nassauw et al., 2001).

Alterations in superoxide dismutase (SOD), peroxidase, and catalase activities, besides tissue glutathione concentrations have been reported in diabetes (Loven et al., 1986). In this sense, reducing oxidative stress with antioxidants may be an effective strategy for reducing the complications associated with the disease (Johansen et al., 2005). Several drugs are used to control blood glucose levels in diabetic patients, with mitigating effects in most cases (Damasceno et al., 2004; Takaku et al., 2006). In general, antioxidant supplementation may improve the quality of life of diabetic patients and prevent associated complications (Pandey et al., 2011). L-glutathione has shown to play an important role in cellular protection against oxidative stress (Jones et al., 1995), preventing or improving diabetes-related complications.

Gastrointestinal disorders are widely observed in diabetes, and neuropathy may be caused by the enteric nervous system (Vincent et al., 2004). The nitration of proteins in enteric neurons could be the cause of enteric neuronal death in DM, and such neuronal death appears to be correlated with increases in oxidative stress (Rivera et al., 2011). The present study evaluated oxidative stress parameters and protein nitration in myenteric neurons in the jejunum of diabetic rats supplemented with L-glutathione.

2. Materials and methods

All of the procedures described in this study conformed with the ethical principles adopted by the Brazilian Society for Laboratory Animal Science (SBCAL) and were approved by the Ethics Committee on Animal Experimentation of the State University of Maringá (UEM).

2.1. Experimental procedure

Adult male Wistar rats (*Rattus norvegicus*) were obtained from the Central of Animal Facilities, UEM. After 88 days of age, the animals were transferred to the vivarium in the Department of Morphological Sciences where they were housed in polypropylene cages (40 cm length × 33 cm width × 17 cm height) with a feeder and water bottle. They were maintained under controlled temperature (22° C) and lighting (12 h/12 h light/dark cycle). Food and water were available *ad libitum*.

After a period of adaptation to the new environment, when the rats were 90 days old, they were available for the experimental period of 120 days. The animals were randomly allocated to four groups ($n = 6$/group): normoglycemic (N), normoglycemic supplemented with L-glutathione (NGT), diabetic (D), and diabetic supplemented with L-glutathione (DGT). The rats in the D and DGT groups underwent the induction of type 1 DM after fasting for 14 h. Diabetes mellitus was induced with an intravenous injection of streptozotocin (35 mg/kg body weight, Sigma, St. Louis, MO, USA) dissolved in 10 mM citrate buffer solution (pH 4.5). To verify the establishment of the experimental model, a drop of blood was obtained from the animal's tail for photometric glucose (glucose-dye-oxidoreductase) determination (Accu-Chek Active glucose meter, Roche Diagnostics GmbH, Mannheim, Germany). All animals submitted to an induction had blood glucose > 200 mg/dl were used in the D and DGT groups.

In the NGT and DGT groups, L-glutathione (Deg, São Paulo, SP, Brazil) was incorporated into the standard laboratory chow at a concentration of 1% (Ueno et al., 2002). Supplementation began from the third day after diabetes induction. Non-supplemented animals (N and D groups) received balanced standard chow (Nuvilab, Colombo, PR, Brazil).

2.2. Material collection and processing

At 210 days of age, the animals were anesthetized with thiopental (40 mg/kg). Blood was collected by cardiac puncture to determine blood glucose levels using the glucose oxidase method (Bergmeyer and Bernet, 1974). Two hours before euthanasia, vincristine sulfate (0.5 mg/kg body weight, Eurofarma Laboratórios, São Paulo, SP, Brazil) was injected. The jejunums of all of the animals were collected, washed in phosphate-buffered saline (PBS; 0.1 M, pH 7.3), and subjected to various procedures.

2.3. Preparation of homogenates

The jejunum (5 mg/ml) was weighed in a solution of monobasic potassium phosphate buffer (K_2HPO_4) in 10 mM of 0.9% sodium chloride (NaCl), pH 7.4. Each sample was then subjected to ultra-Turrax homogenization (Marconi, Piracicaba, SP, Brazil) in an ice bath. The total homogenate was used to measure chemiluminescence (CL). Supernatants were obtained by centrifugation at 11000 rotations per minute at 4° C for 15 min to determine total antioxidant capacity (TAC), catalase activity, and NO production.

2.4. Measurement of tert-butyl hydroperoxide-initiated chemiluminescence

The interaction between reactive oxygen and nitrogen species in biological membranes was evaluated by tert-butyl hydroperoxide (t-butyl)-initiated CL (Gonzalez-Flecha et al., 1991). Samples (800 µl) of the total homogenate were incubated with 180 µl potassium phosphate monobasic buffer at 37 °C for 5 min under light. Afterwards, 20 µl of 3 mM t-butyl was added, which was prepared at the time of use and protected from light. The reaction was monitored on a TD 20/20 Glomax luminometer (Turner Designs, USA). Chemiluminescence was detected at 300–650 nm for 15 min. Increased photon emission indicates the presence of hidro/lipoperoxide in the membranes, revealing oxidative stress in the tissue. The curves were obtained by the interpolation of 15 points (minute by minute) from the original curve. The results are expressed as Relative Light Units (RLU)/g tissue.

2.5. Determination of total antioxidant capacity

Total antioxidant capacity was evaluated by CL according to the technique described by Repetto et al. (1996). This technique evaluates the levels of water-soluble antioxidants in total tissue, especially low-molecular-weight antioxidants, using a reaction medium that contains 20 µM 2,2-azo-bis(2-amidinopropane) (ABAP) and 200 µM luminol. The addition of 70 µl of the supernatant decreases the luminescence of a baseline period (induction time [T_{ind}]) proportional to the amount of antioxidants present in the sample until it reaches the level of light pattern generation. The system was calibrated with Trolox, and the comparison T_{ind} of sample and T_{ind} of Trolox were used to determine TAC in µM Trolox, using the equation: TAC (µM Trolox) = T_{ind} sample/T_{ind} Trolox.

2.6. Determination of catalase activity

Catalase activity was determined using the Aebi (1984) technique. Samples (xx mg/mL) were homogenized in 10 mM phosphate buffer, pH 7.4 and centrifuged at 7500 *g* at 4 °C for 15 min. Supernatant (200 µL) was used in a final 2 mL reaction medium with 10 mM H2O2.