Biomedicine & Pharmacotherapy 105 (2018) 724–733

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

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

Resveratrol promotes neuroprotection and attenuates oxidative and nitrosative stress in the small intestine in diabetic rats

Paulo Emilio Botura Ferreira[a,*], Evandro José Beraldi[b], Stephanie Carvalho Borges[b], Maria Raquel Marçal Natali[b], Nilza Cristina Buttow[b]

[a] Federal University of Pampa, KM 592 BR 472 Road, Uruguaiana, Rio Grande do Sul, 97500-970, Brazil
[b] Departament of Morphological Sciences, State University of Maringá, 5790 Colombo Avenue, Maringá, Paraná, 87020-900, Brazil

ARTICLE INFO

Keywords:
Resveratrol
Neuroprotection
Myenteric plexus
Diabetes
Oxidative stress
Nitrosative stress

ABSTRACT

Damages to the enteric nervous system caused by diabetes mellitus (DM) are frequently attributed to oxidative and nitrosative stress. We aimed to investigate the effect of Resveratrol (RSV) (10 mg/kg) on oxidative and nitrosative stress in the intestinal wall and morphoquantitative aspects of the myenteric plexus of the duodenum, jejunum and ileum in diabetic rats. Twenty-four rats were distributed into four groups (n = 6/group): control (C group), control treated with RSV (CR group), diabetic (D group), and diabetic treated with RSV (DR group) for 120 days. Immunohistochemical staining techniques for the general neuronal population, nitrergic and calretinin neuronal subpopulations, enteric glial cells and glial fibrillary acid protein were performed in the myenteric plexus. Furthermore, parameters of oxidative and nitrosative stress were analyzed in the intestinal wall. RSV attenuated oxidative and nitrosative stress and prevented neuronal loss and hypertrophy of the HuC/D-IR, nNOS-IR and CALR-IR neuronal subpopulations in the DR group compared with the D group ($P < 0.05$). In addition, RSV prevented the increase in glial fibrillary acid protein fluorescence in the DR group compared with the D ($P < 0.05$). These results suggest that RSV has antioxidant and neuroprotective effects in myenteric plexus in rats with experimental DM.

1. Introduction

Diabetic gastrointestinal autonomic neuropathy (DAN) is a complication of chronic diabetes mellitus (DM) [1]. These lesions usually impair sensory and motor gastric function [1,2], leading to bloating, abdominal pain, diarrhea, constipation, and delayed gastric emptying [3,4]. These signs and symptoms are characteristic of lesions in the enteric nervous system (ENS) [5]. In animal models of DAN, damage is commonly seen in the form of morphoquantitative changes in neurons and glial cells [3,5–8]. The pathogenesis of these changes has not been fully elucidated, but oxidative stress that results from diabetes hyperglycemia is one of the main factors that has been reported in the literature [4,9].

Much attention has been given to the use of compounds with antioxidant activity to prevent and treat complications associated with diabetes [5–8,10,11]. Resveratrol (RSV; 3,4',5-trihydroxystilbene) has emerged as a possible treatment candidate because of its powerful antioxidant action, reflected mainly by its ability to scavenge free radicals [9,12] and regenerate endogenous antioxidants [9,13,14]. RSV is a natural polyphenol that is found in high concentrations in the skin and seeds of grapes [13], red wine, peanuts, pistachios, and chocolate [15]. Unlike other antioxidants, the use of high doses of RSV is well tolerated and considered non-toxic [16]. This low toxicity was proven both *in vitro* and *in vivo* in both animals and humans, making RSV a promising candidate for the prevention and treatment of various diseases that are related to oxidative stress [17].

Considering its effect on neurons, RSV has been reported to have a neuroprotective role because to its antioxidant effect in the central nervous system (CNS) in animals with experimental diabetic neuropathy [9,13]. Neurons are extremely vulnerable to oxidative stress because of their high consumption of oxygen and low levels of antioxidant defense enzymes. However, the effects of RSV in the ENS in

Abbreviations: DAN, diabetic gastrointestinal autonomic neuropathy; ENS, enteric nervous system; DM, diabetes mellitus; RSV, resveratrol; STZ, streptozotocin; GFAP, glial fibrillary acidic protein; HuC/D, general neuronal population; nNOS, nitrergic neuronal subpopulation; CALR, nitrergic neuronal subpopulation; S100, glial cells; GSH, glutathione; LOOH, lipid hydroperoxide; SOD, superoxide dismutase; GST, glutathione *S*-transferase; MPO, myeloperoxidase; GB, final glucose blood; TP, total proteins; TG, triacylglycerol; TC, total cholesterol; HDL, HDL cholesterol; LDL, LDL cholesterol; AST, aspartate aminotransferase; ALT, alanine aminotransferase

* Corresponding author at: Campus Uruguaiana, Federal University of Pampa, Road BR 472 KM 592, Uruguaiana, Rio Grande do Sul, 97500-970, Brazil.
E-mail addresses: pauloferreira@unipampa.edu.br (P.E.B. Ferreira), evandro.beraldi@unioeste.br (E.J. Beraldi), scborges@uem.br (S.C. Borges), mrmnatali@uem.br (M.R.M. Natali), ncbuttow@uem.br (N.C. Buttow).

https://doi.org/10.1016/j.biopha.2018.06.030
Received 6 February 2018; Received in revised form 5 June 2018; Accepted 7 June 2018

animals with DAN remain unknown. The objective of the present study was to investigate the effect of RSV (10 mg/kg) on oxidative and nitrosative stress in the intestinal wall and morphoquantitative aspects of the myenteric plexus of the small intestine in rats with experimental DM.

2. Material and methods

2.1. Animals and experimental procedure

All of the animal procedures adhered to the ethical principles adopted by the Brazilian College of Animal Experimentation (COBEA) and were reviewed and approved by the Ethics Committee on Animal Experiments (CEEA) at Universidade Estadual de Maringá (protocol number: 088/2012).

The present study used 24 male Wistar rats at 88 days of age (377.4 ± 7.9 g) that were randomly divided into four groups ($n = 6$/group): normoglycemic control animals treated with vehicle (C group), normoglycemic control animals treated with RSV (CR group), diabetic animals treated with vehicle (D group), and diabetic animals treated with RSV (DR group).

To induce diabetes, the rats in the D and DR groups were fasted for 16 h and then received an intravenous injection (penile vein) of streptozotocin (STZ; Sigma, St. Louis, MO, USA) at a dose of 35 mg/kg body weight, dissolved in 10 mmol/L citrate buffer (pH 4.5). Four days after induction, blood glucose was measured to confirm the establishment of experimental diabetes. Rats that had blood glucose levels > 250 mg/dl were used in the subsequent experiments. The CR and DR groups were treated daily by gavage with RSV at a dose of 10 mg/kg of body weight previously diluted in 20% ethanol (v/v). The choice of the doses of 10 mg/kg of RSV was made based in in several studies that used these same concentrations of RSV and obtained beneficial results [9,14,18–20]. To minimize possible interference from the vehicle, the C and D groups received a daily a 20% ethanol solution (v/v).

2.2. Gastrointestinal transit time

Twenty-four hours before euthanasia, the animals were placed in individual cages with access to food and water. The rats received 0.2 mL of a non-absorbable marker (5% Carmine Red, 1% ethylcellulose) (Corantec, São Paulo, São Paulo, Brasil) by oral gavage to evaluate gastrointestinal transit time. The results are expressed as the latency to the appearance of the first red fecal pellet.

2.3. Blood and tissue collection

At the end of the 120-day experimental period, the rats were weighed and euthanized following anesthesia with Thiopental® (100 mg/kg, i.p.) (Abbott Laboratories, Chicago, IL, USA). Blood was collected and centrifuged, and the supernatants were frozen at −80 °C for the biochemical assays. Laparotomy was then performed to collect the duodenum, jejunum, and ileum for the immunofluorescence experiments. The jejunum and ileum samples were also used for nitrite estimation. The jejunum samples were also used to evaluate oxidative and inflammatory parameters.

2.4. Biochemical assays

Plasma final glucose (GB; it refers to the level of fasting glucose that the animals presented at the end of the experimental period), total proteins (TP), triacylglycerol (TG), total cholesterol (TC), high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, and the activity of the enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were assayed using commercial kits (Bioliquid®, Analisa®, São Paulo, São Paulo, Brazil) and determined using a spectrophotometer (Bioplus2000, São Paulo, São Paulo, Brazil). High-density lipoprotein cholesterol was calculated using the Friedewald formula [21].

2.5. Tissue processing for immunofluorescence

The duodenum, jejunum, and ileum were collected from all of the animals, washed with phosphate-buffered saline (PBS; 0.1 M, pH 7.3), and carefully filled with 4% paraformaldehyde fixative solution (pH 7.4) (Sigma-Aldrich, São Paulo, São Paulo, Brazil). All of the samples were then maintained for 3 h in the same solution at 4 °C. After fixation, all of the segments were cut into smaller segments (approximately 1 cm^2) that were subsequently microdissected under a stereomicroscope to obtain whole mounts from the muscle layer.

2.6. Immunofluorescence

Three whole mounts were obtained from each intestinal segment. The first section was double-labeled to reveal immunoreactivity for the general neuronal population (HuC/D-IR) and nitric oxide synthase (nNOS-IR) immunoreactivity for the nitrergic subpopulation. A second section was stained to reveal immunoreactivity for the glial cells (S100-IR) and glial fibrillary acidic protein (GFAP-IR) immunoreactivity. A third section was stained to reveal calretinin protein (CALR) immunoreactivity. All of these procedures for the immunofluorescence assays were performed according to Ferreira et al. [5] and the antibodies used were described in Table 1.

2.7. Quantitative analysis of immunoreactive myenteric neurons and glial cells

HuC/D-immunoreactive (IR), nNOS-IR, S100-IR, and CALR-IR myenteric neurons were quantified using images that were randomly obtained from whole mounts in each animal. The images were captured with a 20× objective (200× magnification of the image) using a high-resolution AxioCam camera (Zeiss, Jena, Germany) coupled to an Axioskop Plus light microscope (Zeiss) and digitized on a computer using AxioVision Release 4.1 software. All of the neurons and glial cells that were present in 32 images were manually identified and counted using ImagePro Plus 4.5.0.29 image analysis software (Media Cybernetics, Silver Spring, MD, USA). The area of each image was approximately 0.35 mm^2, and the total quantified area was 11.2 mm^2. The results are expressed as neurons or glial cells per cm^2. All of the

Table 1
Primary and secondary antibody used for immunohistochemistry.

Primary antibody	Host	Dilution	Company	Secondary antibody	Dilution	Company
HuC/D	Mouse	1:500	Molecular Probes, Invitrogen	Anti-mouse Alexa Fluor 488	1:500	Molecular Probes, Invitrogen
nNOS	Rabbit	1:500	Zymed	Anti-rabbit Alexa Fluor 546	1:500	Molecular Probes, Invitrogen
S-100	Rabbit	1:300	Molecular Probes, Invitrogen	Anti-rabbit Alexa Fluor 488	1:400	Molecular Probes, Invitrogen
GFAP	Goat	1:500	Abcam	Anti-goat Alexa Fluor 546	1:500	Molecular Probes, Invitrogen
CALR	Goat	1:1200	Abcam	Anti-goat Alexa Fluor 488	1:500	Molecular Probes, Invitrogen

HuC/D: RNA binding protein (General population), nNOS: Neuronal nitric oxide synthase, S-100: Glial cells, GFAP: Glial fibrillary acid protein, CALR: Calcium binding protein.