Available online at www.sciencedirect.com

ScienceDirect

Journal of Nutritional Biochemistry 24 (2013) 2152–2157

Journal of Nutritional Biochemistry

Role of the inhibition of oxidative stress and inflammatory mediators in the neuroprotective effects of hydroxytyrosol in rat brain slices subjected to hypoxia reoxygenation☆,☆☆

Susana Cabrerizo[a], José Pedro De La Cruz[a,*], Juan Antonio López-Villodres[a], Javier Muñoz-Marín[a], Ana Guerrero[a], José Julio Reyes[a], María Teresa Labajos[b], José Antonio González-Correa[a]

[a]*Laboratorio de Investigaciones Antitrombóticas e Isquemia Tisular (LIAIT), Department of Pharmacology and Therapeutics*
[b]*Faculty of health Sciences, School of Medicine, University of Málaga, Spain*

Received 8 February 2013; received in revised form 1 July 2013; accepted 12 August 2013

Abstract

The aim of this study was to analyze the mechanism of the neuroprotective effect of hydroxytyrosol (HT) in an experimental model of hypoxia-reoxygenation in rat brain slices. After reoxygenation the increase in lactate dehydrogenase efflux was inhibited by HT in a concentration-dependent manner and dose-dependent inhibition after oral administration to rats for 7 days (1, 5 and 10 mg/kg per day). Maximum inhibition was 57.4% in vitro and 38.7% ex vivo. Hydroxytyrosol reduced oxidative stress parameters: it inhibited lipid peroxidation and increased enzymatic activities related with the glutathione system both in vitro and after oral administration to rats. The increase in prostaglandin E_2 and interleukin 1β after reoxygenation were inhibited after incubation of brain slices with HT and after oral administration. The accumulation of nitric oxide in brain slices was reduced in a concentration-dependent manner. In conclusion, HT exerts a neuroprotective effect in a model of hypoxia-reoxygenation in rat brain slices, both in vitro and after 7 days of oral administration to rats. HT exerts an antioxidant activity and lowered some inflammatory markers in this model.

Keywords: Hydroxytyrosol; Hypoxia-reoxygenation; Inflammation; Neuroprotection; Oxidative stress

1. Introduction

Hydroxytyrosol (2-(3,4-dihydroxyphenylethanol) is a natural antioxidant [1,2] derived from the chemical [3] or enzymatic [4] hydrolysis of glycoside oleuropein. It is present in large amounts in all parts of olive trees (*Olea europaea*).

Scientific evidence from several sources has accumulated on the beneficial effects of the Mediterranean diet in preventing cardiovascular disease [5,6] and some types of cancer [7,8], and in reducing overall mortality [9]. The benefits of the Mediterranean diet have been attributed, in part, to the antioxidant effect of some of its components [10]. A key component of this diet is olive oil, which contains polyphenol compounds such as hydroxytyrosol (HT) which has a clear antioxidant effect [11].

There is emerging evidence for the preventive effect of the Mediterranean diet on ischemic stroke [12,13]. Although the neuroprotective effect of virgin olive oil administration in healthy rats has been documented [14,15], information remains insufficient regarding the possible neuroprotective effect against brain damage of HT, its main polyphenolic component. However, an HT extract prepared from olive mill wastewater (45.5% of hydroxytyrosol) was shown to reduce neuronal damage induced by ferrous salts or sodium nitroprusside as inducers of oxidation [16]. Moreover, both HT and HT acetate showed a neuroprotective effect in rat brain slices subjected to hypoxia reoxygenation, an effect that has been attributed mainly to its antioxidant properties [17].

The aim of the present study was to examine the neuroprotective effect of HT in rat brain slices subjected to an experimental model of hypoxia reoxygenation both in vitro and after oral administration in rats. In addition to oxidative stress, other biochemical pathways of neuronal damage in the ischemic process, such as inflammatory

Abbreviations: GSH, reduced glutathione; GSHpx, glutathione peroxidase; GSHtf, glutathione transferase; GSSG, oxidized glutathione; HT, hydroxytyrosol; IL-1β, interleukin 1β; LDH, lactate dehydrogenase; MDA, malondialdehyde; NO^-_2 + NO^-_3, nitrites + nitrates; PGE_2, prostaglandin E_2; TBARS, thiobarbituric acid reactive substances.

☆ Conflict of interest: None of the authors declares any potential conflicts of interest.

☆☆ Role of the funding source: This study was partially supported by two grants (AGL-04-7935-C03-02 and AGL7-66373-C04) from Programa Consolider-Ingenio from the Spanish Ministry of Education and Science (CICYT).

* Corresponding author. Department of Pharmacology, School of Medicine. University of Málaga, Campus de Teatinos s/n, 29071 Málaga, Spain. Tel.: +34 952 131567; fax: +34 952 131568.

E-mail address: jpcruz@uma.es (J.P. De La Cruz).

0955-2863/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.jnutbio.2013.08.007

prostaglandin, nitrite accumulation and interleukin production, were studied in this experimental model.

2. Materials and methods

2.1. Materials

Lactate dehydrogenase reagent kits were obtained from Biosystem SA (Barcelona, Spain). Hydroxytyrosol [2-(3,4-dihydroxyphenyl)ethanol] was obtained from Cayman Chemical (Ann Arbor, MI, USA); the purity was ≥98%, according to the manufacturer (Fig. 1). All other reagents were from Sigma Chemical (St. Louis, MO, USA).

2.2. Study design

The experimental animals (10 rats per group) were 2-month-old adult male Wistar rats (body weight 200–250 g). All rats were used in accordance with current Spanish legislation for animal care, use and housing (RD 223/1998, based on European Directive 86/609/CEE). The recommendations in Guide for the Care and Use of Laboratory Animals (NIH publication No. 86–23, revised 1985) were followed, as well the Spanish Law on the Protection of Animals, where applicable. The study protocol was approved by the University of Malaga Ethics Committee for the Use of Animals.

In the in vitro experiments, rats (10 brains per experiment) were anesthetized with pentobarbital sodium (40 mg/kg i.p.), then they were decapitated with a guillotine. Hydroxytyrosol was incubated in the experimental buffer (see below). In the ex vivo experiments rats were distributed randomly into four groups of 10 animals each: (A) control group animals treated with daily saline p.o. for 7 days, (B), animals treated with 1 mg/kg per day p.o. HT for 7 days, (C) animals treated with 5 mg/kg per day p.o. HT for 7 days, (D) animals treated with 10 mg/kg per day p.o. hydroxytyrosol for 7 days. Chow and water were self-administered ad libitum. Either saline or HT was administered from 9:00 to 10:00 a.m. The last dose was administered 1 h before the animal was killed.

2.3. Sample processing

At the end of day 30 all animals from all groups were anesthetized with pentobarbital sodium (40 mg/kg i.p.). The rats were decapitated with a guillotine and the brain was removed.

2.4. In vitro model of rat brain hypoxia reoxygenation

We used a previously described in vitro method of hypoxia-reoxygenation in brain slices [18]. The cortex and midbrain were cut coronally into 1-mm slices with a vibrating microtome (Capdem Instruments, San Francisco, CA, USA). The slices were placed in buffer (composition in mmol/L: 100 NaCl, 0.05 KCl, 24 $NaHCO_3$, 0.55 KH_2PO_4, 0.005 $CaCl_2$, 2 $MgSO_4$, 9.8 glucose, pH 7.4) and perfused with a mixture of 95% O_2 and 5% CO_2. After 30 min to reach equilibrium, the slices were placed in fresh buffer of the same composition except that the concentration of $CaCl_2$ was 3 mmol/L, that of $MgSO_4$ was 0.001 mmol/L, and no glucose was included. This solution was perfused with a mixture of 95% N_2 and 5% CO_2 for 20 min (hypoxia). Then the slices were placed in fresh buffer containing glucose and the solution was perfused with a mixture of 95% O_2 and 5% CO_2 (reoxygenation).

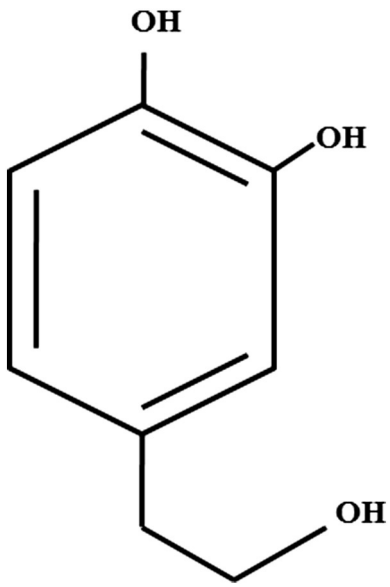

Fig. 1. Chemical structure of hydroxytyrosol.

Brain slices (two per analytical test) were analyzed: (1) after 30 min of incubation and before N_2 perfusion, (2) after 20 min of perfusion with N_2, and (3) after 180 min of reoxygenation. For all studies the tissues were quickly frozen in liquid nitrogen and stored at −80 °C until the day of the experiment, which was done not more than 7 days after the sample was frozen.

2.5. Analytical techniques

All techniques were run in a single-blind manner, i.e., the persons who did the assays were unaware of the origin and nature of the samples.

2.5.1. Lipid peroxidation

To quantify lipid peroxidation we measured thiobarbituric acid reactive substances (TBARS) in brain cell membrane-enriched fractions [19]. Absorbance was determined spectrophotometrically at 532 nm (FLUOstar-POLAEstar. BMG Labtechnologies. Offenburg. Alemania). The results were expressed as micromoles of TBARS per milligram of protein.

2.5.2. Glutathione levels

Total glutathione was measured spectrofluorometrically [20]. Brain tissue was homogenized in 0.1 M sodium phosphate buffer (pH 8.0) with 25% phosphoric acid at a proportion of 1:20, then centrifuged at 13 000×*g* for 15 min at 4 °C to obtain the supernatant. Cuvettes were prepared for spectrofluorometry with sodium phosphate buffer, the supernatant for each sample, and *o*-phthaldehyde. To determine the proportions of oxidized and reduced glutathione we incubated the supernatant from each sample with 4-vinylpyridine, then proceeded for total glutathione.

2.5.3. Enzyme activities related to glutathione

Glutathione peroxidase (GSHpx) and glutathione transferase (GSHtf) were determined with a spectrophotometric kinetics method [21,22]. Brain samples were diluted in 0.1 M phosphate-buffered saline (pH 7.0) and 25% phosphoric acid. The mixture was homogenized and centrifuged at 13 000 g for 15 min at 4 °C. The supernatant was used to determine protein concentration after neutralization with 0.1 N NaOH, and to determine the enzyme activities described below.

Glutathione peroxidase. A volume equivalent to 25 μg protein was taken from each supernatant, and 0.1 M phosphate-buffered saline was added to a volume of 880 μL, together with 53 μL glutathione reductase, 133 μL GSH, 100 μL nicotinamide-adenine dinucleotide phosphate (NADPH) and 100 μL terbutyl-hydroperoxide. The preparation was read at 340 nm and the decrease in absorbance was recorded every 30 s for 5 min.

Glutathione transferase. One hundred microliters of GSH was added to the volumes of sample and buffer indicated above for glutathione peroxidase, and the test was carried out in the same way.

2.5.4. Lactate dehydrogenase assay

Tissue damage was measured by examining lactate dehydrogenase (LDH). Enzyme activity was measured spectrophotometrically at 340 nm with a commercial kit (Cytotoxicity Detection Kit, Roche Applied Science, Barcelona, Spain) according to the manufacturer's instructions.

2.5.5. Prostaglandin E_2

Brain tissues were snap-frozen in liquid nitrogen and stored until analysis. The samples were homogenized (1:10 wt/vol) in 15% methanol with 0.1 N phosphate-buffered saline (pH 7.5), then centrifuged at 37 000×*g* for 15 min at 4 °C. The supernatant was run through a C18 column (Bio-Rad Laboratories, Hercules, CA, USA) that had been activated with absolute methanol, followed by washing with distilled water. After the sample had been assayed, the column was washed with 15% methanol in distilled water followed by petroleum ether. Prostaglandins were eluted with methylformate. The samples were then dried at room temperature under a nitrogen current and reconstituted with phosphate-buffered saline. The concentration of prostaglandin E2 was measured with a commercial enzyme immunoassay (GE Healthcare UK Limited, Little Chalfont, Buckinghamshire, UK).

2.5.6. Nitrite + nitrate

As an indirect indicator of overall nitric oxide production, we determined nitrite + nitrate levels in the incubation buffer. One milliliter of buffer was filtered through Ultrafree MC microcentrifuge filters to remove high-molecular-weight substances released by cell lysis. The nitrite + nitrate level was measured with a commercial kit (Cayman Chemical Company) based on the Griess reaction, after the nitrates were converted to nitrites with nitrate reductase. Levels of nitrite + nitrate were measured spectrophotometrically at 540 nm and compared with a standard curve obtained with sodium nitrite.

2.5.7. Interleukin 1β

The tissue was homogenized in a volume of 1 mL of a buffer (4 °C, pH 7.2) containing 50 mM Tris, 1 mM EDTA, 6 mM $MgCl_2$, 1 mM phenylmethylsufonyl fluoride, 5 μg/mL leupeptin, 1 mg/mL antipain, 1 mg/mL aprotinin and 1 mg/mL soybean trypsin inhibitor. After mixing, the samples were transferred to silicone tubes, then sonicated (VC-50 T Vibracell, Sonics Materials, Newtown, CT, USA) for 5–10 s and centrifuged at