Biochimica et Biophysica Acta 1862 (2016) 1827–1838

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Signaling mechanisms underlying the glioprotective effects of resveratrol against mitochondrial dysfunction

Bruna Bellaver *, Larissa Daniele Bobermin, Débora Guerini Souza, Marília Danielly Nunes Rodrigues, Adriano Martimbianco de Assis, Moacir Wajner, Carlos-Alberto Gonçalves, Diogo Onofre Souza, André Quincozes-Santos *

Departamento de Bioquímica, Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

ARTICLE INFO

Article history:
Received 17 February 2016
Received in revised form 2 June 2016
Accepted 29 June 2016
Available online 01 July 2016

Keywords:
Resveratrol
Mitochondrial dysfunction
Heme oxygenase-1 (HO-1)
Hippocampal astrocytes
Nuclear factor kappa B (NFκB)

ABSTRACT

Resveratrol, a polyphenol found in grapes and red wine, exhibits antioxidant, anti-inflammatory, anti-aging and, neuroprotective effects. Resveratrol also plays a significant role modulating glial functionality, protecting the health of neuroglial cells against several neuropsychiatric in vivo and in vitro experimental models. Mitochondrial impairment strongly affected astrocyte functions and consequently brain homeostasis. Molecules that promote astrocyte mitochondrial protection are fundamental to maintain brain energy balance and cellular redox state, contributing to brain healthy. Thus, the present study was designed to evaluate some glioprotective mechanisms of resveratrol against mitochondrial damage promoted by azide exposure in hippocampal primary astrocyte cultures. Azide treatment provoked deleterious effects, including the dysfunction of mitochondria, the deterioration of redox homeostasis, the augmentation of pro-inflammatory cytokines and impairment of glutamate uptake activity. However, resveratrol prevented these effects, protecting hippocampal astrocytes against azide-induced cytotoxicity through the heme-oxygenase-1 (HO-1) pathway and inhibiting p38 mitogen-activated protein kinase (p38 MAPK) and nuclear factor kappa B (NFκB) activation. Resveratrol also protected astrocytes via phosphatidylinositide 3-kinase (PI3K)/Akt. These results contribute to the comprehension of the mechanisms by which resveratrol mediates hippocampal astrocyte protection against mitochondrial failure and implicate resveratrol as an important glioprotective molecule.

1. Introduction

Mitochondrial failure is recognized as a common feature encountered in brain aging and many neurological diseases, particularly in the pathogenesis of neurodegenerative disorders [1–3]. The dysfunction of mitochondrial energy metabolism reduces ATP production, followed by the generation of reactive oxygen species (ROS). The decreased activity of complex IV in the electron transport chain has been detected in the brains of patients with ischemia, epilepsy, and Alzheimer's and Huntington's diseases [2,4,5]. Accordingly, sodium azide is a rapid inhibitor of cytochrome *c* oxidase, reflecting the frequent use of this molecule to induce acute oxidative stress.

Resveratrol (3,5,4′-trihidroxy-*trans*-stilbene) is a polyphenol commonly detected in a variety of dietary sources, including grapes, peanuts and wine. The neuroprotective effects of resveratrol might reflect the antioxidant, anti-inflammatory and anti-aging properties of this compound [6–11]. This phytoalexin has been implicated in the protection of astrocytes and neurons against in vivo and in vitro experimental models [12–16]. Recently, we demonstrated that the protective effects of resveratrol against an inflammatory challenge in astrocytes might be associated with heme oxygenase-1 (HO-1), p38 mitogen-activated protein kinase (p38 MAPK) and extracellular signal–regulated kinase (ERK) pathways [17]. Although the protective role of resveratrol in the central nervous system (CNS) has been well established, the mechanisms of the effects mediated by astrocytes have not been fully clarified.

Astrocytes are the most abundant glial cell type in the CNS, and these cells participate in a wide range of functions to maintain brain homeostasis [18–20]. Following brain damage, these cells become reactive, triggering a cascade of events, which play a key role in the recruitment of other glial cells, strongly affecting neuronal survival [21–23]. In conditions of oxidative injury and inflammation, the astrocytic synthesis

* Corresponding authors at: Departamento de Bioquímica, Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade, Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600 — Anexo, Bairro Santa Cecília, 90035-003, Porto Alegre, RS, Brazil.
E-mail addresses: brunabellaver90@gmail.com (B. Bellaver), andrequincozes@ufrgs.br (A. Quincozes-Santos).

http://dx.doi.org/10.1016/j.bbadis.2016.06.018
0925-4439/

of HO-1 is increased, playing an important role in neuroprotection. The crucial role of HO-1 in responding to CNS alterations has generated renewed interest in the regulation and function of this enzyme [24–26]. Therefore, the pathophysiological roles of astrocytes have become the primary focus in the investigation of numerous diseases and have emerged as a target for preventive/therapeutic strategies for these diseases. In this context, these studies have demonstrated the detrimental impact of azide exposure on astrocytic functionality, but the role of resveratrol against mitochondrial failure in these cells remains unclear.

Considering the oxidant imbalance mediated through azide, the key role of astrocytes responding to this event and the glioprotective functions of resveratrol, including the anti-inflammatory and antioxidant activities of this compound, the aim of the present study was to characterize the effects of resveratrol as a glioprotective agent on azide-induced mitochondrial dysfunction in hippocampal astrocyte primary cultures. We also investigated the involvement of HO-1, nuclear factor kappa B (NFκB), p38 MAPK and phosphatidylinositide 3-kinase (PI3K)/Akt signaling pathways in these effects. We hypothesized was that resveratrol would prevent mitochondrial damage induced by azide via the modulation of glial antioxidative, anti-inflammatory and glutamatergic functions.

2. Material and methods

2.1. Chemicals

Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12), other cell cultures materials, NFκB p65 ELISA, JC-1, TRIzol Reagent, SYBR green PCR master mix, PCR master mix were purchased from Gibco/Invitrogen (Carlsbad, CA, USA). Resveratrol, sodium azide, DNase, GSH Standard Stock Solution, *o*-phthaldialdehyde, *c*-glutamylhydroxamate, ZnPP IX and LY294002 were obtained from Sigma-Aldrich (St. Louis, MO, USA). L-[H^3]-glutamate was obtained from Amersham. The TNF-α ELISA was obtained from PeproTech (Rocky Hill, NJ, USA). IL-1β was purchased from eBioscience (USA). RANSOD and RANSEL were obtained from Randox (Autrim, UK). All other chemicals were purchased from common commercial suppliers.

2.2. Animals

Male Wistar rats were obtained from our breeding colony (Department of Biochemistry, UFRGS, Porto Alegre, Brazil), maintained under a controlled environment (12 h light/12 h dark cycle; 22 ± 1 °C; ad libitum access to food and water. The animals received regular laboratory chow, Nuvilab-CR1, from Nuvital, Brazil). All animal experiments were performed in accordance with the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals and Brazilian Society for Neuroscience and Behavior recommendations for animal care. The experimental protocols were approved by the Federal University of Rio Grande do Sul Animal Care and Use Committee (process number 27,543).

2.3. Primary hippocampal astrocyte cultures

This protocol was in accordance with Bellaver et al. [17,27]. Briefly, the hippocampi from newborn (1–2 days old) male Wistar rats were aseptically removed from the cerebral hemispheres. The tissue was enzymatically digested (with trypsin 0.05%) and mechanically dissociated, followed by centrifugation at 100 g for 5 min. The cells were resuspended in Hanks' balanced salt solution (HBSS) containing DNase (0.003%) and subjected to decantation for 20 min. Supernatant was collected and centrifuged for 7 min (400 g). The cell pellet was resuspended in DMEM/F12 [10% FBS, 15 mM HEPES, 14.3 mM $NaHCO_3$, 1% Fungizone and 0.04% gentamicin], plated onto 6- or 24-well plates pre-coated with poly-L-lysine at a density of $3–5 \times 10^5$ cells/cm^2. The cells were cultured at 37 °C in an atmosphere with 5% CO_2. The first medium exchange occurred 24 h after obtaining the culture. The medium change occurred once every two days during the 1st week and once every four days during the 2nd week. The purity of the primary astrocyte cultures was assessed by immunocytochemistry for glial fibrillary acidic protein (GFAP). OX-42 (CD11b/c) and NeuN were used as microglial and neuronal markers, respectively. Under these conditions, cell cultures were confirmed as >98% positive for GFAP, indicating an astrocytic phenotype. Furthermore, approximately 2% of the astrocyte cell cultures showed OX-42-positive staining.

2.4. Cellular treatments

Resveratrol was dissolved in ethanol and appropriately diluted prior to astrocyte treatment. After dilution, the final concentration of ethanol was 0.25%, which did not affect cell viability. The concentration dependency of resveratrol and azide on cell viability and membrane integrity was evaluated after incubating the cells with the indicated concentrations of resveratrol (1, 10 and 100 μM) and/or azide (1, 5 and 10 mM) for a range of periods (1, 3, 6 and 24 h) (data not shown). These results are consistent with previous publications [6,28]. When the cells reached confluence (approximately 10 days in vitro), the culture medium was exchanged with serum-free DMEM/F12, and the cells were pre-incubated in the absence or presence of 100 μM of resveratrol for 1 h. After pre-incubation, the resveratrol treatment was maintained and 5 mM azide was added for 3 h. During all procedures, the cells were maintained at 37 °C in an atmosphere with 5% CO_2. To study the involvement of the HO-1 and phosphatidylinositide 3-kinase (PI3K)/Akt signaling pathways in the effects of resveratrol on azide-induced oxidative damage, we coincubated 10 μM of ZnPP IX (a HO-1 inhibitor) or 10 μM of LY294002 (a PI3K/Akt inhibitor), with resveratrol. Under these conditions, ZnPP IX and LY294002 did not change the HO-1 and PI3K/Akt levels (data not shown).

2.5. RNA extraction and quantitative RT-PCR

Total RNA was isolated from astrocyte cultures using TRIzol Reagent according to manufacturer's instructions (Invitrogen, Carlsbad, CA). The concentration and purity of the RNA were spectrophotometrically determined at a ratio of 260/280. Subsequently, 1 μg of total RNA was reverse transcribed using the Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) in a 20 μL reaction, according to the manufacturer's instructions. The mRNAs of glutamate-aspartate transporter (GLAST) and glutamate transporter-1 (GLT-1) were quantified using the TaqMan real-time RT-PCR system with inventory primers and probes purchased from Applied Biosystems (Foster City). The mRNA quantification of cytochrome *c* oxidase (EC 1.9.3.1), cyclooxygenase-2 (COX-2, EC 1.14.99.1), NFκB, HO-1, PI3K and Akt was performed using primer pairs (Table 1) and Power SYBR Green PCR Master Mix (Invitrogen). Quantitative RT-PCR was performed in duplicate using the Applied Biosystems 7500 Fast system. No-template and no-reverse transcriptase controls were included in each assay, producing no detectable signal during the 35–40 cycles of amplification. Target mRNA levels were normalized to β-actin levels using the $2^{-\Delta\Delta Ct}$ method [29].

2.6. Mitochondrial membrane potential — ΔΨm (JC-1 assay)

To determine the ΔΨm, the cells were incubated for 30 min with JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3'tetraethylbenzimidazolylcarbocyanine iodide, 2 μg/mL) [30]. The cells were subsequently homogenized and centrifuged, washed once with HBSS, and transferred to a 96-well plate. Fluorescence was measured using an excitation wavelength of 485 nm and emission wavelengths of 540 and 590 nm. The ΔΨm was calculated using the ratio of 590 nm (red fluorescent J-aggregates) to