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Oxidative stress mediated by NMDA, AMPA/KA channels in acute hippocampal slices: Neuroprotective effect of resveratrol

André Quincozes-Santos *, Larissa Daniele Bobermin, Ana Carolina Tramontina, Krista Minéia Wartchow, Bárbara Tagliari, Diogo Onofre Souza, Angela T.S. Wyse, Carlos-Alberto Gonçalves

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

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ABSTRACT

Glutamate is the major excitatory neurotransmitter in the brain and over-stimulation of the glutamate receptors, NMDA, AMPA and kainate (KA), may cause neuronal death in epilepsy, seizures and neurodegenerative diseases. Mitochondria have critical cellular functions that influence neuronal excitability, such as regulation of Ca^{2+} homeostasis and ATP production to maintain Na^+K^+-ATPase in the central nervous system (CNS). However, mitochondria are also the primary site of reactive oxygen species (ROS) production, and oxidative stress can induce cellular damage. Resveratrol, a polyphenol found in grapes and wines, presents antioxidant and neuroprotective effects on brain pathologies. This study sought to determine the neuroprotective effect of resveratrol against glutamate toxicity in acute hippocampal slices, using specific inhibitors of glutamate channels, and to investigate the targets of glutamate excitotoxicity, such as mitochondrial membrane potential ($\Delta\Psi_m$), Na^+K^+-ATPase and glutamine synthetase (GS) activity. Resveratrol decreases intracellular ROS production, most likely by mechanisms involving NMDA, AMPA/KA, intracellular Ca^{2+} and the heme oxygenase 1 (HO1) pathway, and prevents mitochondrial dysfunction and impairments in Na^+K^+-ATPase and GS activity after glutamate activation. Taken together, these results show that resveratrol may exhibit an important neuroprotective mechanism against neuropsychiatric disorders, focusing on mitochondrial bioenergetics and oxidative stress, as well as inhibitory effects on ionic channels.

1. Introduction

Hippocampal slices have been widely used to investigate the electrophysiological and biochemical parameters of the Central Nervous System (CNS), due to partially preserved neuron-astrocyte circuitry with a suitable control of extracellular medium (Nardin et al., 2009; Thomazi et al., 2004, 2008). Morphological studies have confirmed the integrity of a large number of synaptic terminals, including glial cells (Nagy and Li, 2000). The use of brain slices does have some limitations that are inherent in the use of isolated neuronal and/or glial cells (Aitken et al., 1995; de Almeida et al., 2008; Nagy and Li, 2000; Nardin et al., 2009).

Glutamate is the major excitatory neurotransmitter in the brain (Danbolt, 2001). Extracellular glutamate is normally kept at low levels by Na^+-dependent transport into glia and neurons (Anderson and Swanson, 2000; Danbolt, 2001). Excessive glutamate stimulation or persistent activation of glutamate-gated ion channels may cause neuronal degeneration in experimental models of epilepsy, seizures, ischemia, trauma and neurodegenerative diseases (Coyle and Puttfarcken, 1993; Friedman et al., 1994; Gupta et al., 2002; Maragakis and Rothstein, 2006). Ionotropic glutamate receptors, N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate (KA), are the predominant receptors at most excitatory synapses (Coyle and Puttfarcken, 1993). The overactivation of these receptors and disrupted glutamate homeostasis has been recognized to play a prominent role in the development and generation of neurological diseases (Lau and Tymianski, 2010). Furthermore, activation of an NMDA receptor under depolarizing conditions permits Ca^{2+} to flow through its channels, thus increasing the influx of Ca^{2+} and leading to neuronal degeneration (Carmignoto et al., 1998; Coyle and Puttfarcken, 1993; Ding et al., 2007).

Oxidative stress and mitochondrial metabolic dysfunction are involved in neuronal death (Halliwell, 2006). Mitochondria have critical cellular functions that influence neuronal excitability, including production of adenosine triphosphate (ATP), fatty acid oxidation, neurotransmitter biosynthesis, and regulation of Ca^{2+}

* Corresponding author. Address: 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. Tel.: +55 51 3308 5559; fax: +55 51 3308 5535.
E-mail address: andrequincozes@yahoo.com.br (A. Quincozes-Santos).

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homeostasis (Halliwell, 2007; Rosenstock et al., 2004; Waldbaum and Patel, 2010). The majority of ATP produced by the mitochondria is utilized to maintain Na^+K^+-ATPase in the CNS (Lingrel, 1992; Waldbaum and Patel, 2010). However, mitochondria are the primary site of reactive oxygen species (ROS) production. ROS overproduction generates oxidative stress, and this condition can affect the functioning of cellular macromolecules, such as lipids, proteins and nucleic acids (Halliwell, 2006, 2007). Glutamate exposure may induce overactivation of NMDA-type glutamate receptors and may increase Ca^{2+} influx into neurons. This Ca^{2+} can be taken up by mitochondria, where it may stimulate the generation of oxidative/nitrosative species that damage the mitochondria and the cell (Nicholls, 2008).

Resveratrol (3,5,4′-trihydroxy-trans-stilbene), a redox active compound, is a phytoalexin found in a wide range of dietary sources, including grapes, peanuts and wine (especially red wine); it exhibits antioxidant, anti-inflammatory, cardioprotective, estrogenic and antitumoral activities (Baur and Sinclair, 2006; Bobermin et al., 2012; Park et al., 2012; Pervaiz, 2003; Quincozes-Santos and Gottfried, 2011; Yang et al., 2013). Resveratrol also presents neuroprotective properties and has been investigated in several neurodegenerative models, such as epilepsy, stroke and Alzheimer's and Parkinson's diseases (Bastianetto and Quirion, 2010; Baur and Sinclair, 2006; Fukui et al., 2010; Kim et al., 2007; Kumar et al., 2007; Shetty, 2011). Although the neuroprotective role of resveratrol in the CNS is well established, the cellular mechanisms underlying resveratrol-induced neuroprotection must be better elucidated (Fukui et al., 2010; Lee et al., 2010).

Classically, glutamate exposure induces activation of NMDA and AMPA/KA receptors, mitochondrial damage, and, consequently, oxidative stress (Coyle and Puttfarcken, 1993). These events are associated with neuropsychiatric disorders. Thus, in this study, the biochemical parameters and probable mechanisms by which resveratrol protects hippocampal slices from glutamate excitotoxicity were evaluated using specific inhibitors of glutamate-gated channels. Additionally, glutamate excitotoxicity targets were investigated, such as mitochondrial membrane potential ($\Delta\Psi_m$), Na^+K^+-ATPase and glutamine synthetase (GS) activity.

2. Materials and methods

2.1. Animals

Male Wistar rats (30 days old) were obtained from our breeding colony (Department of Biochemistry), maintained under controlled environment (12 h light/12 h dark cycle, 22 ± 1 °C, *ad libitum* access to food and water). All animal experiments were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Federal University of Rio Grande do Sul Animal Care and Use Committee (process number 21215).

2.2. Materials

Resveratrol, chemical reagents, anti-Na^+K^+-ATPase α-1 and α-3 subunits, anti-β-actin, MTT Formazan, 2′-7′-dichorofluorescein diacetate (DCFH) and γ-glutamylhydroxamate were purchased from Sigma (St. Louis, MO, USA). JC-1 was purchased from Gibco/Invitrogen (Carlsbad, CA, USA). Anti-HO1 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals were purchased from local commercial suppliers.

2.3. Preparation and incubation of hippocampal slices

Animals were killed by decapitation, the brains were removed and placed in cold saline medium with the following composition (in mM): 120 NaCl; 2 KCl; 1 $CaCl_2$; 1 $MgSO_4$; 25 HEPES; 1 KH_2PO_4 and 10 glucose, adjusted to pH 7.4 and previously oxygenated for 15 min with O_2. The hippocampi were dissected and transverse slices of 0.3 mm were obtained using a McIlwain Tissue Chopper. Slices were then transferred immediately into 24-well culture plates, each well containing 0.3 mL of physiological medium and only slice. The medium was changed every 15 min with fresh saline medium at room temperature (maintained at 25 °C). Following a 120 min equilibration period, the medium was removed and replaced with fresh saline containing, or not, 100 μM resveratrol for 30 min at 30 °C. After this period, the medium was maintained and 1 mM glutamate was added to the medium for 30 min at 30 °C in a water bath. For all analyzed parameters, the results obtained with vehicle (0.25% ethanol) were not different from those obtained under control conditions without ethanol.

2.4. Membrane integrity and metabolic activity assays

2.4.1. Lactate dehydrogenase assay

The lactate dehydrogenase (LDH) assay was conducted in 50 μL of extracellular medium using a commercial colorimetric assay from Doles (Brazil). Results are expressed as percentages of the control value.

2.4.2. Lactate release assay

The lactate release assay was conducted in 100 μL of extracellular medium using a commercial colorimetric assay from Labtest (Brazil). Results are expressed as percentages of the control value.

2.4.3. MTT reduction assay

Slices were treated with 0.5 mg/mL of MTT for 30 min at 30 °C. The MTT formazan was dissolved in DMSO. Absorbance values were measured at 560 and 650 nm. Results are expressed as percentages of the control value.

2.5. Intracellular ROS production

DCFH oxidation was used to measure intracellular ROS production (Quincozes-Santos et al., 2009). DCFH-DA (2′-7′-dichlorofluorescein diacetate) is hydrolyzed by intracellular esterases to dichlorofluorescin (DCFH), which is trapped within the cell. This non-fluorescent molecule is then oxidized to fluorescent dichlorofluorescin (DCF) by action of cellular oxidants. To test whether NMDA and AMPA/KA activation, intracellular Ca^{2+} signaling, NO and heme oxygenase 1 (HO1) pathways were involved in the effect of resveratrol against glutamate-induced increase ROS production, we used the following specific inhibitors: Dizocilpine (MK801) – 10 μM, 6-cyano-7-nitroquinoxaline-2-3-dione (CNQX) – 10 μM, 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM) – 10 μM, N^{ω}-nitro-L-arginine methyl ester (L-NAME) – 500 μM, and Zinc Protoporphyrin IX (ZnPP IX) – 10 μM, for 30 min added together with resveratrol. After glutamate incubation, slices were treated with DCFH-DA (10 μM) for 30 min at 30 °C. The fluorescence was measured in a plate reader (Spectra Max GEMINI XPS, Molecular Devices, USA) with excitation at 485 nm and emission at 520 nm (Quincozes-Santos et al., 2009). The ROS production was calculated as Unit of Fluorescence – UF/mg protein and was expressed as percentage of control.

2.6. Western blot analysis

Slices were homogenate using lysis solution with 4% SDS, 2 mM EDTA, 50 mM Tris–HCl, pH 6.8. Equal amounts of proteins from each sample were boiled in sample buffer [62.5 mM Tris–HCl, pH 6.8, 2% (w/v) SDS, 5% β-mercaptoethanol, 10% (v/v) glycerol,

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