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Ammonia-induced oxidative damage in neurons is prevented by resveratrol and lipoic acid with participation of heme oxygenase 1

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ABSTRACT

Ammonia is a metabolite that, at high concentrations, is implicated in neurological disorders, such as hepatic encephalopathy (HE), which is associated with acute or chronic liver failure. Astrocytes are considered the primary target of ammonia toxicity in the central nervous system (CNS) because glutamine synthetase (GS), responsible for ammonia metabolism in CNS, is an astrocytic enzyme. Thus, neuronal dysfunction has been associated as secondary to astrocytic impairment. However, we demonstrated that ammonia can induce direct effects on neuronal cells. The cell viability was decreased by ammonia in SH-SY5Y cells and cerebellar granule neurons. In addition, ammonia induced increased reactive oxygen species (ROS) production and decreased GSH intracellular content, the main antioxidant in CNS. As ammonia neurotoxicity is strongly associated with oxidative stress, we also investigated the potential neuroprotective roles of the antioxidants, resveratrol (RSV) and lipoic acid (LA), against ammonia toxicity in cerebellar granule neurons. RSV and LA were able to prevent the oxidative damage induced by ammonia, maintaining the levels of ROS production and GSH close to basal values. Both antioxidants also decreased ROS production and increased GSH content under basal conditions (in the absence of ammonia). Moreover, we showed that heme oxygenase 1 (HO1), a protein associated with protection against stress conditions, is involved in the beneficial effects of RSV and LA in cerebellar granule neurons. Thus, this study reinforces the neuroprotective effects of RSV and LA. Although more studies *in vivo* are required, RSV and LA could represent interesting therapeutic strategies for the management of HE.

1. Introduction

Ammonia is a metabolite that, at high concentrations, is implicated in neurological disorders, such as hepatic encephalopathy (HE), which is associated with acute or chronic liver failure (Albrecht and Jones, 1999; Haussinger and Schliess, 2008; Felipo, 2013; Butterworth, 2014). Ammonia crosses the blood–brain barrier readily (Ott and Larsen, 2004) and, in the central nervous system (CNS), the predominant route for its metabolism is the amidation of glutamate to form glutamine *via* glutamine synthetase (GS), an enzyme located in the astrocytes (Norenberg and Martinez-Hernandez, 1979; Cooper, 2011). This feature makes the astrocytes the primary target of ammonia-induced damage, and neuronal dysfunction has been associated as secondary to astrocytic impairment (Norenberg, 1998; Rangroo Thrane et al., 2013).

However, *in vitro* and *in vivo* evidence shows that ammonia induces direct effects on neuronal cells (Yang et al., 2004; Klejman et al., 2005; Rangroo Thrane et al., 2013; Chen et al., 2014). Moreover, NMDA receptor activation and downstream effects, *e.g.* reactive oxygen and nitrogen (ROS/RNS) production, seem to be related to ammonia neurotoxicity (Kosenko et al., 2000; Hilgier et al., 2003; Zielińska et al., 2003; Giordano et al., 2005). Thus, glutathione (GSH) represents a major antioxidant in the CNS (Dringen, 2000), preventing ammonia toxicity in neurons (Klejman et al., 2005).

Antioxidants are substances that delay, prevent or reverse oxidative damage to a target molecule (Gutteridge and Halliwell, 2010). A great number of molecules with antioxidant activity have

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demonstrated neuroprotective roles, such as resveratrol (RSV) and lipoic acid (LA) (Shay et al., 2009; Albarracin et al., 2012; Pallàs et al., 2013; Virmani et al., 2013). RSV (3,5,4′-trihydroxy-trans-stilbene) is a naturally occurring polyphenol present in grapes, berries and also red wines (Baur and Sinclair, 2006). The protective effects of RSV in the brain have been studied in a variety of pathological events, including tumors (Leone et al., 2008; Gagliano et al., 2010; Filippi-Chiela et al., 2011), ischemic injury (Raval et al., 2008; Shin et al., 2012; Wang et al., 2014) and neurodegenerative disorders (Vingtdeux et al., 2008; Huang et al., 2011; Richard et al., 2011). RSV presents important antioxidant properties, possibly by its direct scavenging effect and/or activation of cellular antioxidant defenses (Bastianetto et al., 2014).

LA is another compound with beneficial effects that is synthesized in small amounts by plants and animals, including humans, and is an essential cofactor for mitochondrial enzymes (Haramaki et al., 1997; Smith et al., 2004). When exogenously administrated, it is a potent modulator of the cell redox status and presents anti-inflammatory effect (Perera et al., 2011; Kleinkauf-Rocha et al., 2013; Rocamonde et al., 2013; Tomassoni et al., 2013). Thus, LA has emerged as a potential therapeutic agent in pathologies involving oxidative stress.

Heme oxygenase 1 (HO1) is the rate-limiting enzyme in the pathway in which pro-oxidant heme is degraded into the antioxidants biliverdin and bilirubin and carbon monoxide (CO) (Yoshida and Kikuchi, 1974; Maines, 2004). The inducible HO1 isoform is activated not only by its physiological substrate heme, but also by various stress and noxious conditions, such as oxidative stress, hypoxia and inflammation (Doré, 2005; Ryter et al., 2006; Pae et al., 2008; Jazwa and Cuadrado, 2010). In the CNS, HO1 has been reported to operate an important cytoprotective/defense mechanism for cells exposed to oxidant challenges (Le et al., 1999; Scapagnini et al., 2004). Interestingly, this enzyme can participate in the positive effects of antioxidants RSV and LA (Sakata et al., 2010; Yamada et al., 2011; Kim et al., 2013; Koriyama et al., 2013; Lin et al., 2013; Quincozes-Santos et al., 2013).

Previously, we demonstrated that both RSV and LA exert beneficial effects in astroglial cells exposed to ammonia, avoiding oxidative stress, proinflammatory cytokine release and impairment of important astroglial functions, such as glutamate uptake, GS activity and GSH levels (Bobermin et al., 2012, 2013). Here, we investigated the potential neuroprotective roles of the antioxidants, RSV and LA, as well as the protective effects of HO1 against oxidative damage-mediated ammonia toxicity on SH-SY5Y cells and cerebellar granule neurons.

2. Materials and methods

2.1. Chemicals

Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12), Minimum Essential Media (MEM), fetal bovine serum (FBS) and other materials for cell cultures were purchased from Gibco (Carlsbad, CA, USA). DNase, poly-D-lysine, resveratrol, lipoic acid, ZnPP IX, methylthiazolyldiphenyltetrazolium bromide (MTT), 2′-7′-dichlorofluorescein diacetate (DCFH-DA), GSH standard and *o*-phthaldialdehyde were obtained from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals were purchased from common commercial suppliers.

2.2. SH-SY5Y cell culture

The human neuroblastoma cell line, SH-SY5Y, obtained from the American Type Culture Collection (ATCC; USA), was cultured in DMEM/F12 (pH 7.4) containing 10% FBS, 15 mM HEPES, 14.3 mM $NaHCO_3$, 1% amphotericin B and 0.032% gentamicin, at 37 °C, in a humidified atmosphere of 5% CO_2/95% air. When cells reached approximately 90% confluence, they were sub-cultured using 0.05% trypsin/ethylene-diaminetetracetic acid (EDTA) and seeded in 24-well plates (6×10^4 cells/well) (Lopes et al., 2010).

2.3. Cerebellar granule neuron culture

Cerebellar granule neuron cultures were prepared from 7-day-old Wistar rats, as previously described with some modifications (Boeck et al., 2005). Animals were obtained from our breeding colony (Department of Biochemistry, UFRGS, Porto Alegre, Brazil) and maintained in a controlled environment (12-h light/12-h dark cycle; 22 ± 1 °C; *ad libitum* access to food and water). The 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). Briefly, cerebella were aseptically dissected and dissociated enzymatically (with trypsin 0.05%) and mechanically, in Krebs-Ringer buffer containing DNAse (0.003%). After decantation for 20 min, the supernatant was collected and centrifuged for 5 min (1000 rpm). The cells from the pellet were resuspended in MEM supplemented with 10% FBS, 14.3 mM $NaHCO_3$, 25 mM KCl and 0.032% gentamicin, plated in 6- or 24-well plates pre-coated with poly-D-lysine (10 μg/mL) at a density of $3–10 \times 10^5$ cells/well and maintained at 37 °C in a humidified atmosphere of 5% CO_2/95% air. The non-neuronal cells were inhibited by the addition of 20 μM cytosine arabinofuranoside 20–24 h after seeding. These cultures contain >90% granule cells and a small number of glial (2–3%) and endothelial cells (<1%) (Kingsbury et al., 1985).

2.4. Experimental treatments

SH-SY5Y cells were treated when they reached approximately 75% confluence. The culture medium was replaced by DMEM/F12 1% FBS and cells were incubated with ammonia at indicated concentrations (1–10 mM) for 24 h. Cerebellar granule neurons were treated at 7 days *in vitro* (DIV) with ammonia (1–10 mM) for 24 h. To study the effects of antioxidants against ammonia neurotoxicity, cells were pre-treated for 1 h with resveratrol (RSV – 10 μM) or lipoic acid (LA – 10 μM) and 2 mM ammonia was then added for 24 h. During all treatments, the cells were maintained at 37 °C in an atmosphere of 5% CO_2/95% air. To study the role of HO1 in the effects of antioxidants, cells were co-incubated with ZnPP IX (10 μM), a specific inhibitor of HO1.

2.5. MTT assay

SH-SY5Y cells and cerebellar granule neurons were incubated with 50 μg/mL MTT for 3 h or 0.5 h, respectively, at 37 °C in 5% CO_2/95% air. Subsequently, the medium was removed and the MTT crystals were dissolved in dimethylsulfoxide (DMSO). Absorbance values were measured at 560 nm and 650 nm. The results are expressed as percentages relative to control conditions.

2.6. DCFH oxidation

Intracellular ROS levels were detected using DCFH-DA. DCFH-DA was added to the medium at a concentration of 10 μM and cells were incubated for 30 min at 37 °C. Following DCFH-DA exposure, the cells were scraped into phosphate-buffered saline with 0.2% Triton X-100. 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). Values were obtained as fluorescence units/mg protein and expressed as percentages relative to the control conditions.