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Primary cultured astrocytes from old rats are capable to activate the Nrf2 response against MPP+ toxicity after tBHQ pretreatment

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ABSTRACT

Astrocytes are key players for brain physiology, protecting neurons by releasing antioxidant enzymes; however, they are also susceptible to damage by neurotoxins. Nuclear factor erythroid-derived 2-like 2 (Nrf2) is a central regulator of the antioxidant response, and therefore, pharmacologic inducers are often used to activate this transcription factor to induce cellular protection. To date, it still remains unknown if cells from aged animals are capable of developing this response. Therefore, the purpose of this work was to determine if cortical astrocytes derived from old rats are able to respond to tertbuthyl-hydroquinene (tBHQ) pretreatment and stimulate the Nrf2-antioxidant response pathway to induce an antioxidant strategy against MPP+ toxicity, one of the most used molecules to model Parkinson's disease. Our results show that, although astrocytes from adult and old rats were more susceptible to MPP+ toxicity than astrocytes from newborn rats, when pretreated with tertbuthyl-hydroquinene, they were able to transactivate Nrf2, increasing antioxidant enzymes and developing cellular protection. These results are discussed in terms of the doses used to create protective responses.

1. Introduction

Aging is the main risk factor for numerous neurodegenerative disorders, and even though their accurate etiology is largely unknown, oxidative stress has been proposed as one of the primary causes that links the aging process with the establishment of most neuropathies (Simonian and Coyle, 1996; Reynolds et al., 2007), not only through the structural and functional alterations that reactive oxygen species (ROS) produce to cell biomolecules, but also because they are potential mediators of cell death by either necrosis or apoptosis (Friedlander, 2003).

Astrocytes are the most abundant glial cell type, representing more than 50% of the total cortical cells (Dringen, 2000). They are known to be important modulators of brain physiology, particularly during regenerative or protective processes, by producing and releasing several antioxidant enzymes like superoxide dismutase and glutathione precursors, which in turn support neuronal survival and stability (Kahlert and Reiser, 2004; Takuma et al., 2004). Additionally, astrocytes regulate the synaptic transmission as part of the tripartite synapse; maintain the blood–brain barrier integrity, brain cholesterol levels, and copper homeostasis (Scheiber and Dringen, 2013; Kim and De Vellis, 2005). Moreover, it is known that these cells decrease their neuroprotective capacity during aging, thereby playing critical roles in neurodegenerative diseases, because astrocytes are involved in responses to damage and stress in a multifactorial manner, by synthesizing and secreting cytokines and chemokines (Sofroniew and Vinters, 2010). This response is called reactive astrogliosis (Ting et al., 2009) and may be either

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harmful or beneficial because reactive astrocytes can exert both pro- and anti-inflammatory effects. Under pathologic conditions, the development of the pro-inflammatory phenotype might explain the relevance of astroglial cells in the genesis of degenerative processes in the brain (Zhang et al., 2010).

Modifications in redox state are known to modulate transcription factors (Forman et al., 2004; Jones, 2008), such as the nuclear factor erythroid-derived 2-like 2 (Nrf2). Nrf2 is a central regulator of antioxidant and phase II detoxifying enzymes. This transcription factor is an ubiquitous cytosolic protein that is continuously degraded during cellular homeostasis; however, in response to modifications in cellular redox state, Nrf2 is released from its repressor (Keap-1), phosphorylated and translocated into the nucleus, where it binds to the antioxidant response element (ARE) and induces the expression of enzymes such as γGCS and GST, which in turn are related to glutathione (GSH) metabolism (Kraft et al., 2004; Lee et al., 2003). GSH is one of the most intensively studied intracellular nonprotein-thiols because of the critical role it plays in cell biochemistry and physiology. Through maintenance of protein sulfhydryls in the appropriate redox state, GSH regulates important death and/or survival pathways. Redox changes, induced by an altered GSH and/or GSSG balance, also modulate Nrf2 release from Keap-1, and changes in GSH homeostasis have been implicated in the etiology and progression of a number of human diseases (Fernández-Checa and García-Ruiz 2008; Darlington, 2005).

Phenols like curcumin, resveratrol, and tertbuthyl-hydroquinene (tBHQ) are well-known Nrf2 inducers in neurons and astrocytes, and have been widely used to activate the antioxidant response in both cell types (Erlank et al., 2011; Thimmulappa et al., 2002). However, it remains unknown if cells from aged animals are still capable of developing an antioxidant response in reply to such Nrf2 inducers, as it is expected for astrocytes from newborn and adult rats. Hence, the purpose of this study was to determine if astrocytes derived from old rats are able to recruit Nrf2-associated responses and evoke an antioxidant protection against an acute toxic insult. One of the most used molecules to model neurodegenerative diseases, in particular Parkinson's disease, is MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine). MPTP is oxidized in glial cells, mainly in astrocytes, throughout the action of monoamine oxidase B into MPP+ (1-methyl-4-phenylpyridinium), which is further incorporated to the dopaminergic neurons where it impairs mitochondrial function, induces ROS generation and cell demise (Przedborski and Vila, 2003; Zhang et al., 2010).

Although its is generally accepted that neurons are vulnerable to the toxic actions of MPTP because of their ability to accumulate and retain MPP+, there are also some reports where MPP+ has been shown to directly exert damage on cultured astrocytes from rats. For example, it has been shown that MPP+ causes impaired energy in astrocytes by affecting mitochondrial function (Di Monte et al., 1992; Chen et al., 2008), MPP+ is concentrated by the mitochondria, where it inhibits complex I activity at the same site as the respiratory inhibitor rotenone (Krueger et al., 1990; Schapira, 2008). In addition, it has been suggested that MPP+ toxicity in cultured astrocytes depends on oxidative and nitrergic stress (Schapira, 2008; Tsai and Lee, 1998). This evidence was preceded by comparative studies demonstrating a differential ability to accumulate MPP+ and express toxicity between rats and mice, suggesting that cultured astrocytes from the first species accumulate less MPP+ while express toxicity at higher concentrations (Tsai and Lee, 1994). Previous data from our group (Alarcón-Aguilar et al., 2014) showed that astrocytes isolated from 24-month-old rats were more susceptible to MPP+ toxicity that astrocytes from newborn and adult (9-month-old) rats. tBHQ is a known Nrf2 inductor, which has been proved in several cellular models and in young animals but not much is known of its effect on old animals. Hence, it was interesting to find out if cells derived from old animals, would still be competent to activate the Nrf2 pathway when pretreated with an inductor such as tBHQ. Therefore, in this study astrocytes derived from old animals were pretreated with tBHQ before the MPP+ insult to determine if old cells are capable to activate Nrf2 protective responses. Our data indicate that when astrocytes derived from old rats were pretreated with tBHQ, they were able to transactivate Nrf2, increasing the content of antioxidant enzymes, improving redox homesotasis measured by GSH/GSSG ratio, and developing protection against mild MPP+ toxicity, supporting the protective character of this pathway for cell survival.

2. Methods

2.1. Chemicals

All chemicals and reagents were purchased from Sigma Chemical Co (St Louis, MO, USA). The reagents obtained from other sources are detailed throughout the text.

2.2. Animals

Astrocytes were isolated from the frontal cortex of neonatal (1- to 3-day-old), adult (9-month-old), and old (24-month-old) albino Wistar rat brains (*Rattus norvegicus*), provided by the closed breeding colony at the Universidad Autónoma Metropolitana-Iztapalapa. A total of 40 neonatal, 80 adult, and 80 old rats were used throughout the study. Before they were assigned to the experiments, adult animals were housed 5-per-cage in polycarbonate cages and provided with standard commercial rat diet (Harlan 2018S, USA) and water *ad libitum*. All procedures with animals were strictly carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the Principles of the Mexican Official Ethics Standard 062-ZOO-1999.

2.3. Cortical astrocytes isolation and culture

Astrocyte primary cultures were obtained according to a protocol established in previous reports (Alarcón-Aguilar et al., 2014; Lin et al., 2007; McCarthy and De Villes, 1980). Pools of 3 animals were used for neonatal astrocytes, while 4 animals were pooled tougher for adult and old cultures. Cells were maintained routinely in MEM medium supplemented with 10% fetal bovine serum, 5% glutamine, 10% glucose, and 10% penicillin-streptomycin. The medium was replaced every 2–3 days. Cells were grown at 37 °C in 60 mm-diameter plates (Corning, Acton, MA, USA) in an atmosphere of 95% air and 5% CO_2. To ensure that the isolated cells were indeed astrocytes, cells were immunostained using polyclonal antibodies against glial fibrillary acidic protein (GFAP). Under these conditions, cultures were confirmed to contain more than 90% cells positive to GFAP. For immunofluorescence experiments, cells were washed with PBS and fixed with 4% paraformaldehyde for 30 minutes. Immediately thereafter, cells were incubated in blocking buffer (2% bovine serum albumin [BSA], 0.2% nonfat milk, 0.4% Triton X100 in phosphate-buffered saline [PBS]) for 1 hour at room temperature. Cells were then washed and incubated for one more hour with the primary antibody anti-GFAP (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Cells were washed 3 times with PBS-Tween 0.2% and incubated with the secondary antibody (ALEXA FLUOR 594 anti-rabbit dilution 1:200). After 4 more washes, cells were incubated with HOECHST (1 μg/mL) for 5 minutes to stain DNA and mark the nuclei. Cells were washed again twice and mounted with fluorescent mounting medium (DakoCytomation, Glostrup, Denmark). Images were obtained with a confocal microscope OLYMPUS BX-51W1 imaging at 30× with the Mercury Lamp, and 2 filters: U-MWU2 330–385 nm excitation and 420 nm