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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

emission for HOECHST, and U-MRFPHQ 535–555 excitation and 570–625 nm emission for ALEXA FLUOR 594.

2.4. Cell viability

After preserved for 2 weeks under in vitro conditions, astrocytes were reseeded at a cell density of 3×10^5 cells per well on a 24-well multichamber (Corning, Acton, MA, USA). Cells were treated with 10, 25, 50, 75, or 100-μM tBHQ for 24 hours. To determine cellular viability, astrocytes were trypsinized and a 20 μL aliquot was stained with an equal volume of a 0.4% trypan blue physiological solution (trypan blue exclusion). The number of living cells in 10 μL of this suspension was scored using 5 fields of a hemocytometer, under a phase-contrast optical microscope, as described elsewhere (López-Diazguerrero et al., 2006).

2.5. Western blot analysis

Treated and untreated astrocytes were trypsinized and resuspended in lysis buffer M-PER (Pierce Chemical, Rockford, IL, USA) supplemented with proteases inhibitor (Complete; Roche Applied Science, Indianapolis, IN, USA), 1 mM phenyl methyl sulfonyl fluoride (PMSF) and 0.1 mM dithiothreitol (DTT). Cell homogenates were incubated at 4 °C for 5–10 minutes, and centrifuged at 14,000×*g*, 4 °C for 20 minutes. Protein concentration was determined in the supernatants using a commercial Bradford reagent (BioRad, Hercules CA, USA) (Bradford, 1976). Cell lysates were separated on 13% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Invitrogen), and probed with anti-GFAP (A-21282) (Life technologies, Carlsbad, CA, USA), anti-Nrf2 (sc-722), anti-GST (sc-138), or anti-γGCS (sc-22755) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Membranes were washed 3 times with TBS-Tween and incubated with a horseradish peroxidase-conjugated α-mouse IgG secondary antibody (Pierce, Rockford, IL, USA) for 1 hour. After the 3 washes, the blots were developed using a commercial chemiluminescent reagent (Supersignal Pierce, Rockford, IL, USA).

2.6. Immunofluorescence experiments

Astrocytes were washed with PBS and fixed with 4% paraformaldehyde for 30 minutes. Immediately thereafter, cells were incubated in blocking buffer (2% BSA, 0.2% nonfat milk, 0.4% Triton X100 in PBS) for 1 hour at room temperature. Cells were washed and incubated for one more hour with the primary antibody anti-Nrf2 (sc-722), (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Cells were washed 3 times with PBS-Tween 0.2% and were incubated with the secondary antibody (ALEXA 488 dilution 1:200). After 4 more washes, cells were further incubated with DAPI (10 μg/mL) for 10 minutes to stain the DNA and mark the nucleus. Cells were washed twice again and mounted with fluorescent mounting medium (DakoCytomation, Glostrup, Denmark). Single plane images were obtained with a confocal microscope LSM-META-Zeiss Axioplan 2 imaging at 30× with the Diodo Laser 405 nm for DAPI and Ar/ML 458/488/514 nm for ALEXA. A co-localization analysis between Nrf2 and the DNA staining (DAPI) was specially performed using the ZEN 2010 program version 6.0 (Carl Zeiss).

2.7. Electrophoretic mobility shift assay

Nuclear extracts were prepared with Igepal CA-630 according to (Gómez-Quiroz et al., 2008). Protein concentration was determined in the supernatant using a commercial Bradford reagent (BioRad, Hercules, CA, USA) (Bradford, 1976). Nrf2-DNA binding activity was assayed using the Nrf2 consensus oligonucleotide: 5′-TTTTCTGCTGACTCAAGGTCCG-3′ (Kweon et al., 2006) (Promega, Madison, WI, USA). Probe was labeled with T4 polinucleotide kinase (USB, Cleveland, OH, USA) and (γ-^{32}P) ATP (3000 Ci/mmol, MP Biomedical, Irving, CA, USA) and purified using Bio-spin 30 chromatography columns (BioRad, Hercules, CA, USA). The reaction mixture contained nuclear protein extract (20 μg) in 5 μL of incubation buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5 mM ethylenediamine-tetraacetic acid (EDTA), 5 mM β-mercaptoethanol, 20% glycerol, 1 μg dI-dC, and ^{32}P-labeled probe. In competition experiments, 100-fold molar excess of non-labeled oligonucleotide was included in the reaction mixture 5 minutes before addition of the labeled probe. The reactions were electrophoresed on 6% polyacrylamide native gels. Gels were exposed in a Storage Phosphor Screen (Amersham Bioscience, Arlington, IL, USA) and were analyzed after 24 hours in a variable-mode imager (Typhon 9400; Amersham Bioscience), using the software Image Quant TL version 6.0 (Amersham Bioscience).

2.8. GSH and GSSG determination

The fluorometric assay previously described by Galván-Arzate et al. (2005) was used. To determine GSH, cells were homogenized in 3.75 mL EDTA and/or phosphate buffer (pH 8.0) plus 1 mL of H_3PO_3 (25%). Homogenates were centrifuged at 3000×*g* for 15 minutes and supernatants were separated. Five hundred μL of supernatant were added to 4.5 mL of phosphate buffer plus 100 μL of o-phthalaldehyde. To quantify GSSG, the same method was used, except that the 500 μL aliquots were resuspended in 200 μL of N-ethylmaleimide (0.04 M) for 30 minutes, and then 100 μL of the homogenates were mixed with 4.3 mL of NaOH (0.01 N) plus 1.8 mL of phosphate buffer and 100 μL of o-phthalaldehyde. All mixtures were incubated at room temperature for 15 minutes, and their fluorescent signals were recorded in the luminescence spectrometer at 420 nm of emission and 350 nm of excitation wavelengths. Final results were expressed as μg of GSH or GSSG per mg of protein.

2.9. Statistical analysis

All experiments minimally represent 3 independent experiments per group performed by triplicate. Data are reported as mean values ± standard deviation, and results were analyzed using a parametric 1-way analysis of variance followed by Tukey–Kramer test. Multiple levels of confidence ($p < 0.05$, $p < 0.01$, and $p < 0.001$) were considered as statistically significant.

3. Results

3.1. Astrocytes isolated from rats of different ages have differential susceptibility to tBHQ

First, to confirm that the primary cultures obtained were indeed astrocytes, GFAP expression was observed in astrocytes derived from newborn, adult, and old rats. The confocal photomicrographs shown in Fig. 1A confirm that more than 90% of cultured cells were certainly astrocytes, a result that has been previously demonstrated by our group (Alarcón-Aguilar et al., 2014). To simplify terminology, from this point on, astrocytes derived from old rats will be abbreviated as OA, whereas astrocytes from adult and newborn rats will be termed as AA and NA, respectively.

Cells derived from old animals are known to be more susceptible to redox alterations and toxicants (Klamt et al., 2002; Gottfried et al., 2002), and because tBHQ is a redox state modifier that is known to modify the redox state by generating H_2O_2 (Erlank et al., 2011), a tBHQ dose-response curve was performed, to assure that the redox modification were maintained at the physiological and not at the

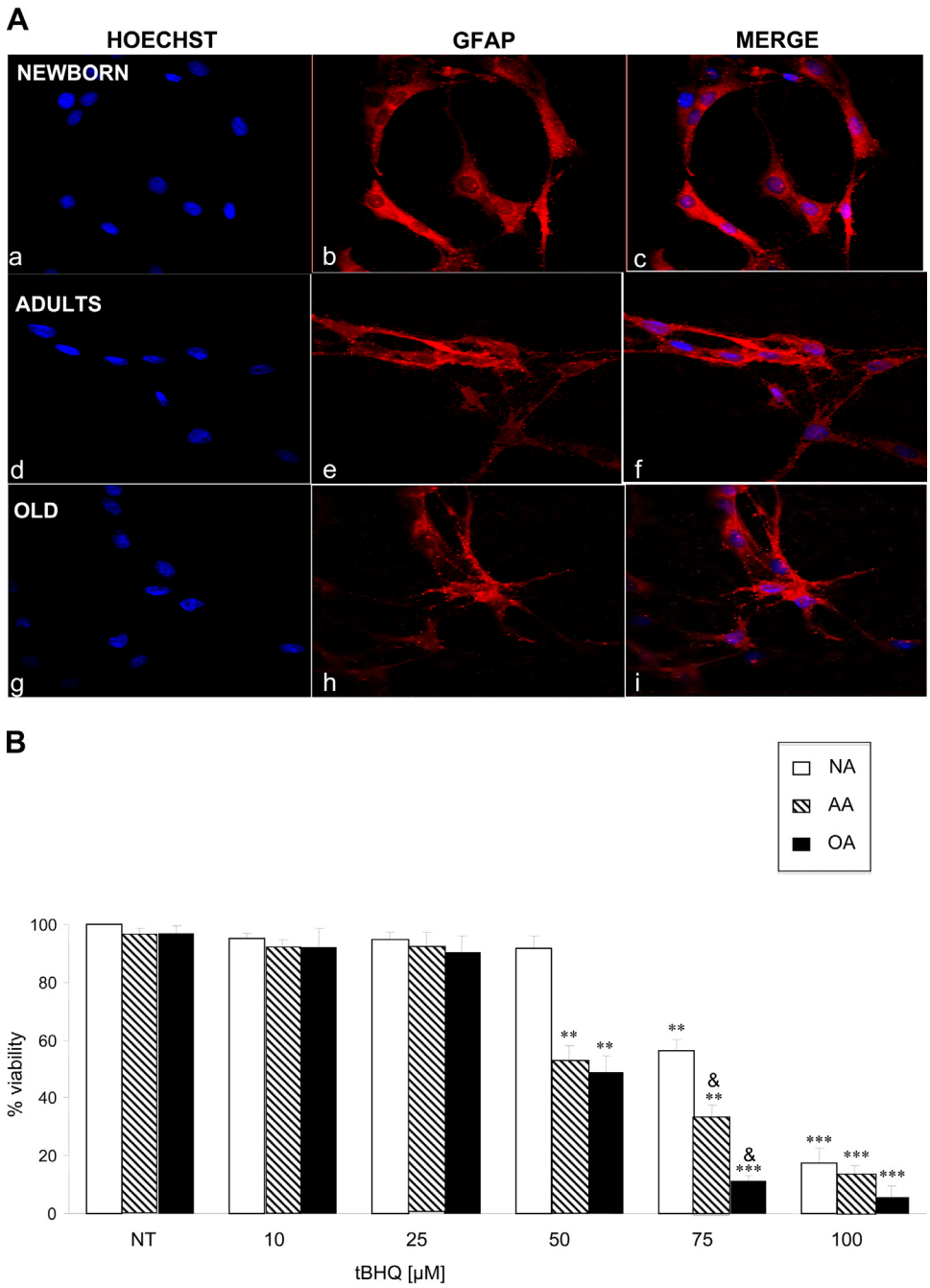

Fig. 1. Representative newborn, adult, and old primary astrocyte staining, and differential susceptibility to tBHQ treatment. (A) Confocal microscopic representative images of astrocyte primary cultures from newborn (NA) (a, b, c), 9-month-old adult rats (AA) (d, e, f), and 24-month-old aged rats (OA) (g, h, i). In a, d and g, hoescht staining (blue); in b, e, and h, GFAP immunostaining (red); in c, f, and i, merge. (B) Primary astrocytes derived from newborn (NA, white bars), adult (AA, shredded bars), and old rats (OA, black bars) were treated with increasing tBHQ concentrations (10, 25, 50, 75, and 100 μM) for 24 hours. Cell viability was assessed using trypan blue as described in section 2, and was normalized against NA as control, which was considered 100%. Each bar represents the mean ± SD of 9 determinations performed in 3 independent experiments. Statistical significance with respect to untreated cells: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ were considered; that is, treated NA, AA, and OA versus untreated NA. Statistical difference between AA and OA versus NA treated with the same tBHQ concentration was designated as $^{\&}$ $p < 0.05$. (C) Primary NA, AA, and OA were treated with different tBHQ concentrations (10, 25, 50 μM) for 24 hours and Nrf2 expression was determined from total protein lysates by Western blot as described in section 2. (D) Densitometric analysis was normalized against actin house keeping, and then against NA as control, which was considered 100%. (E) Each point represents the mean ± SD of 3 determinations performed in independent experiments. NT, nontreated, NA (white bars), AA (shredded bars), and OA (black bars). Statistical significance with respect to untreated cells: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ were considered; that is, treated NA, AA, and OA versus untreated NA. Statistical difference between AA and OA versus NA treated with the same tBHQ concentration was designated as $^{\&}$ $p < 0.05$. Abbreviations: GFAP, glial fibrillary acidic protein; Nrf2, nuclear factor erythroid-derived 2-like 2; SD, standard deviation; tBHQ, tertbuthyl-hydroquinene.

pathologic level. NA, AA, and OA entirely survived to 10 and 25 μM tBHQ 24 hour-treatments (Fig. 1B), while approximately 50% of OA and AA died after the 50-μM treatment. Interestingly, cellular demise with 75 μM was 44% for NA, 65% for AA, and 89% for OA, whereas at 100 μM, more than 80%–90% of the cells from all age groups died.

Along with cellular resistance to this agent, it was important to determine if astrocytes derived from the 3 age groups were able to induce Nrf2 expression. The results presented in Fig. 1C show that all cell cultures increased their Nrf2 content at 24 hours after the 10 and 25 μM tBHQ treatment but only the NA resisted the 50-μM

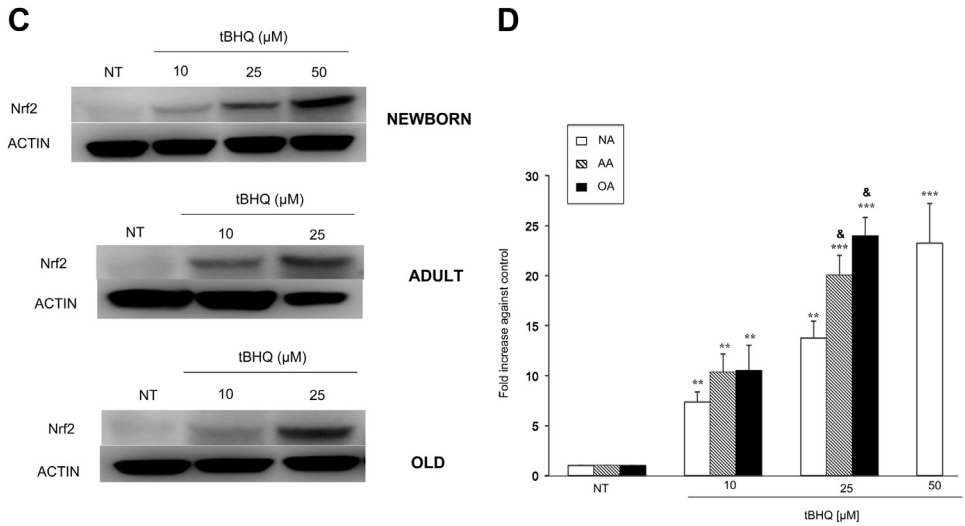

Fig. 1. (*continued*).

treatment and increased Nrf2. No significant differences were found between OA and AA groups at the lower concentrations, indicating that both adult and aged animals might be able to respond to Nrf2 inducers in the same manner than young rats do in that induction range; however, when the tBHQ concentration increased, OA and AA died more rapidly than NA. No astrocytes derived from the different age groups were able to resist the 100-μM tBHQ concentration, all of them died before 24 hours, thereby suggesting that there is a window of efficacy for the pre-conditioning treatment. Based on these results, 25-μM tBHQ was subsequently used to effectively induce the Nrf2-antioxidant response in AA and OA, while 25 or 50 μM tBHQ was used in NA.

3.2. tBHQ induces Nrf2 nuclear translocation

Nrf2 nuclear translocation was assessed by immunofluorescence in a timeline course from the first 30 minutes to 24 hours for the entire duration of tBHQ treatment (0.5, 1, 2, 3, 6, 9, 12, and 24 hours). Panels A (NA), B (AA), and C (OA) in Fig. 2 show some representative images from selected time points to illustrate Nrf2 localization during tBHQ treatment (the remaining time points are not shown). It can be observed that cytosolic Nrf2 levels tend to increase since the begging of the treatment and up to 24 hours, but there is also a high Nrf2 signal in the nucleus since the first hour, which is sustained during the whole treatment, especially in the NA and AA. To better analyze nuclear translocation, Nrf2 co-localization with the DNA marker was determined using the ZEN 2010 program version 6.0; Fig. 2D presents the co-localization in a graphical representation. Interestingly, Nrf2 levels are higher in all time points, when compared with the non-treated cells. The figures also show a dynamic behavior, where Nrf2 translocates in and out of the nucleus. However, it is surprising that this transcription factor remains in the nucleus longer than expected. Still, when the immunofluorescence images are observed, it is also evident that Nrf2 is in the cytosol too, suggesting that besides Nrf2 nuclear translocation, there might also be de novo synthesis, corroborating the Western blots for total Nrf2 in Fig. 1C.

3.3. Nrf2 binds to its DNA response element (ARE) only at early time points

Because Nrf2 was observed in the nucleus for a long time, it was important to determine if it was actually binding to its DNA consensus sequence, the ARE, an electrophoretic mobility shift assay during the same timeline for the tBHQ duration treatment (0.5, 1, 2, 3, 6, 9, 12, and 24 hours) was performed as a functional marker of Nrf2 signaling. The results in Fig. 3 indicate that Nrf2 might migrate to the nucleus as part of a primary response to the change in steady state ROS levels but it only binds to the DNA at early time points. In particular in NA, Nrf2 is attached to the ARE sequence from 30 minutes to 2 hours, with a maximal peak at 1 hour (Fig. 3A), whereas AA have a larger activity window, from 30 minutes to 3 hours (Fig. 3B), and in OA, Nrf2 is apparently only active for a very short time period at 30 minutes (Fig. 3C). So, even if Nrf2 remains in the nucleus for 24 hours (a surprisingly long time), the antioxidant response is only being activated early on time during tBHQ treatment.

As part of the antioxidant defense system commonly activated by Nrf2 through the Nrf2/ARE pathway, GST and γGCS enzymes were evaluated (Fig. 4). Both protein levels increased after tBHQ treatment, although their behavior was quite different. For instance, GST increased between 2.5 and 3.5 times in all age groups, and these results were significantly different from the untreated astrocytes ($p < 0.01$) but no differences were found among the tBHQ concentrations used, nor among the age group. In regard to γGCS, only AA increased their enzymatic content (100%) with 10-μM tBHQ in a significant manner ($p < 0.01$), whereas at 25-μM tBHQ, both AA and OA augmented their γGCS levels (180% and 150%, respectively) ($p < 0.01$). Interestingly, an increase in NA γGCS was only observed at the higher tBHQ concentration, 50 μM (110%) ($p < 0.01$).

3.4. tBHQ pretreatment increases GSH levels and protects old astrocytes against 25 μMPP+ toxicity

Nrf2 induction by tBHQ was found to enhance proteins related to the antioxidant response, even in OA. Therefore the next step was to determine if tBHQ pretreatment protects OA astrocytes against MPP+ toxicity. Previous reports from our group have shown a significant decrease in cellular functionality and viability after 1 hours MPP+ exposure (25 μM and 50 μM) in OA > AA > NA, and this effect was associated to a considerable increase in protein and lipid oxidation. Moreover, OA were found to be significantly more susceptible to MPP+ toxicity (Alarcón-Aguilar et al., 2014). Therefore, those same conditions were used here to challenge tBHQ pretreated astrocytes.

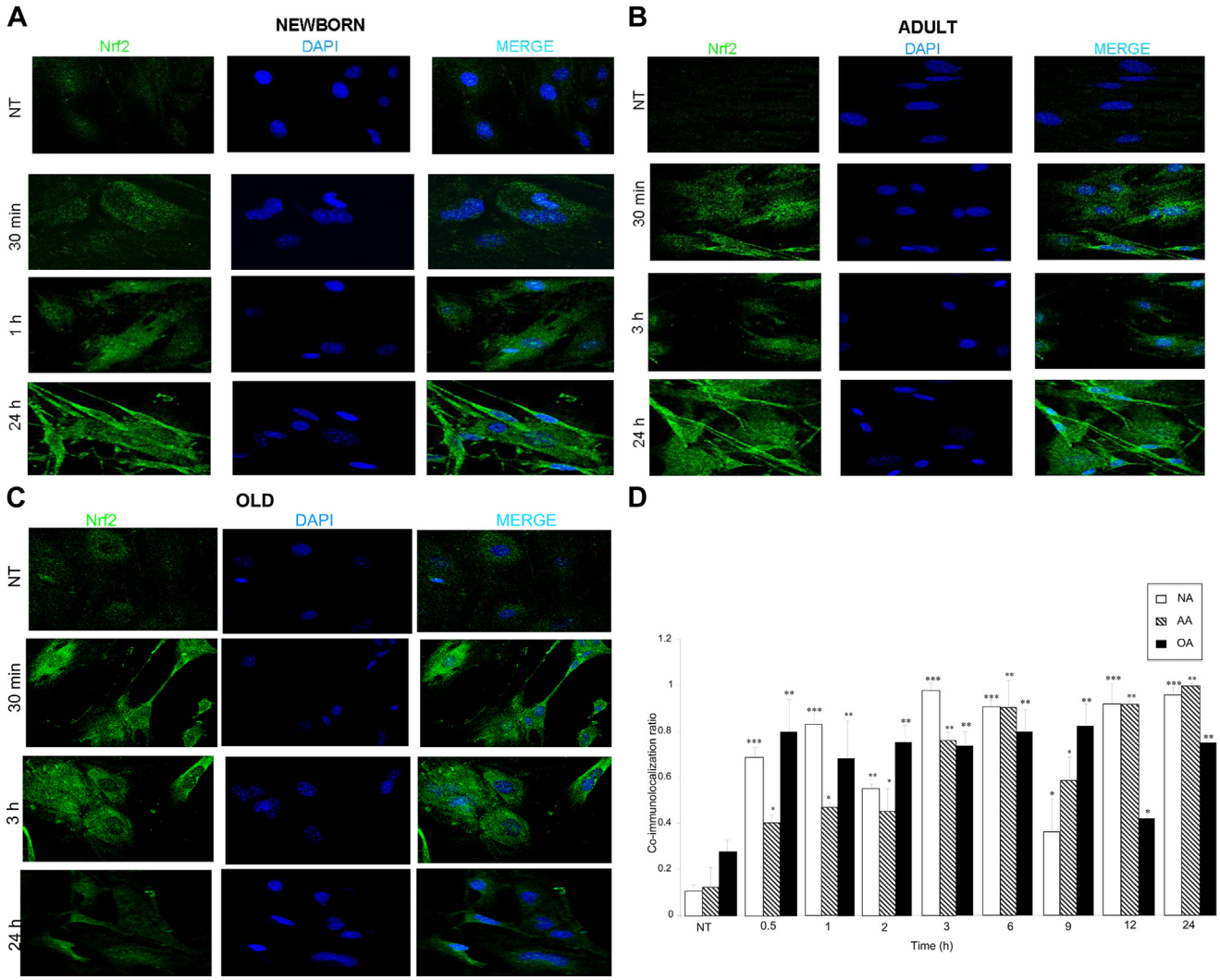

Fig. 2. Nrf2 nuclear translocation after tBHQ treatment. Primary astrocytes were treated with tBHQ for 24 hours. Immunofluorescence was assessed in a timeline course from 0.5, 1, 2, 3, 6, 9 12, and 24 hours during tBHQ treatment. The figure shows 3 representative time points obtained with a confocal microscope LSM-META-Zeiss Axioplan 2 imaging at 30× with the Diodo Laser 405 nm for DAPI (nucleus) and Ar/ML 458/488/514 nm for ALEXA (Nrf2), as described in section 2. (A) Confocal microscopic representative images of astrocyte primary cultures from newborn (NA) at 30 minutes, 1 hour and 24 hours. (B) Confocal microscopic representative images of astrocyte primary cultures from 9-month-old adult rats (AA) at 30 minutes, 3 hours and 24 hours. (C) Confocal microscopic representative images of astrocyte primary cultures from 24-month-old aged rats (OA) at 30 minutes, 3 hours, and 24 hours. (D) Analysis of Nrf2 in the nucleus. The co-immunolocalization of Nrf2 (green) with the DNA marker (DAPI) was done throughout the complete timeline course (0.5, 1, 2, 3, 6, 9 12, and 24 hours) during tBHQ treatment. Co-localization was determined using Zen 2010 program version 6.0. Each point represents the mean ± SD of 3 determinations performed in independent experiments. NA (with bars), AA (shredded bars), and OA (black bars). Statistical significance with respect to each untreated cell type control (NT): * $p < 0.05$), ** $p < 0.01$), *** $p < 0.001$) were considered. Abbreviations: Nrf2, nuclear factor erythroid-derived 2-like 2; SD, standard deviation; tBHQ, tertbuthyl-hydroquinene.

Redox state measured as GSH and/or GSSG was also determined (Fig. 5A–C). When the cells were pretreated with 25 μM tBHQ, GSH increased 2.5 times in OA and 3.5 times in AA, whereas only 10% was observed in NA (Fig. 5A), however a 2.5 increase was observed in NA with 50 μM tBHQ (inset in Fig. 5A). Once the cells were subjected to 25 μM MPP+ toxicity, all cell types changed their redox state to a more oxidized one (Fig. 5C), mainly because of an increase in GSSG (Fig. 5B). Interestingly, OA dramatically augmented their GSSG content (almost 2 times) (Fig. 5B black bars). To determine redox changes induced by tBHQ, cells pretreated with this drug for 24 hours were subjected to an oxidative insult with either 25 μM MPP+ for 1 hour. tBHQ protection against an oxidative shift because of MPP+ toxicity was observed for NA at 50 μM tBHQ, where GSH to GSSG ratio remained similar to the control or untreated astrocytes (Fig. 5C). Noteworthy, AA highly increased GSH levels (shredded bars in Fig. 5A), leading the redox state to a reduced status, even more reduced than the control astrocytes (shredded bars in Fig. 5C) ($p < 0.001$). Finally, as mentioned previously, MPP+ severely increased GSSG levels in OA (black bars in Fig. 5B), thus the increase in GSH brought on by tBHQ stimulation (black bar in Fig. 5A) was effective to sustain normal redox levels. To verify the physiological effect and actual tBHQ/Nrf2/ARE pathway activation for cell protection against an oxidative insult, cellular viability was determined in 25 μM tBHQ pretreated astrocytes and then exposed to 25 μM MPP+. Fig. 6 shows the percentage of viable cells after these conditions. AA presented a significant survival rate of 45% when compared with non-tBHQ pretreated astrocytes, while the protection against cell death in OA was around 60% ($p < 0.05$). Consistently with the previous data, NA pretreated with 25 μM tBHQ did not show an important protective effect, but when pretreated with 50 μM tBHQ a 30% protection was observed (insert in Fig. 6). These results show that even so OA are more susceptible to toxins (cell death observed at 25 μM MPP+: 30% NA, 38% AA, 52% OA), tBHQ pretreatment was

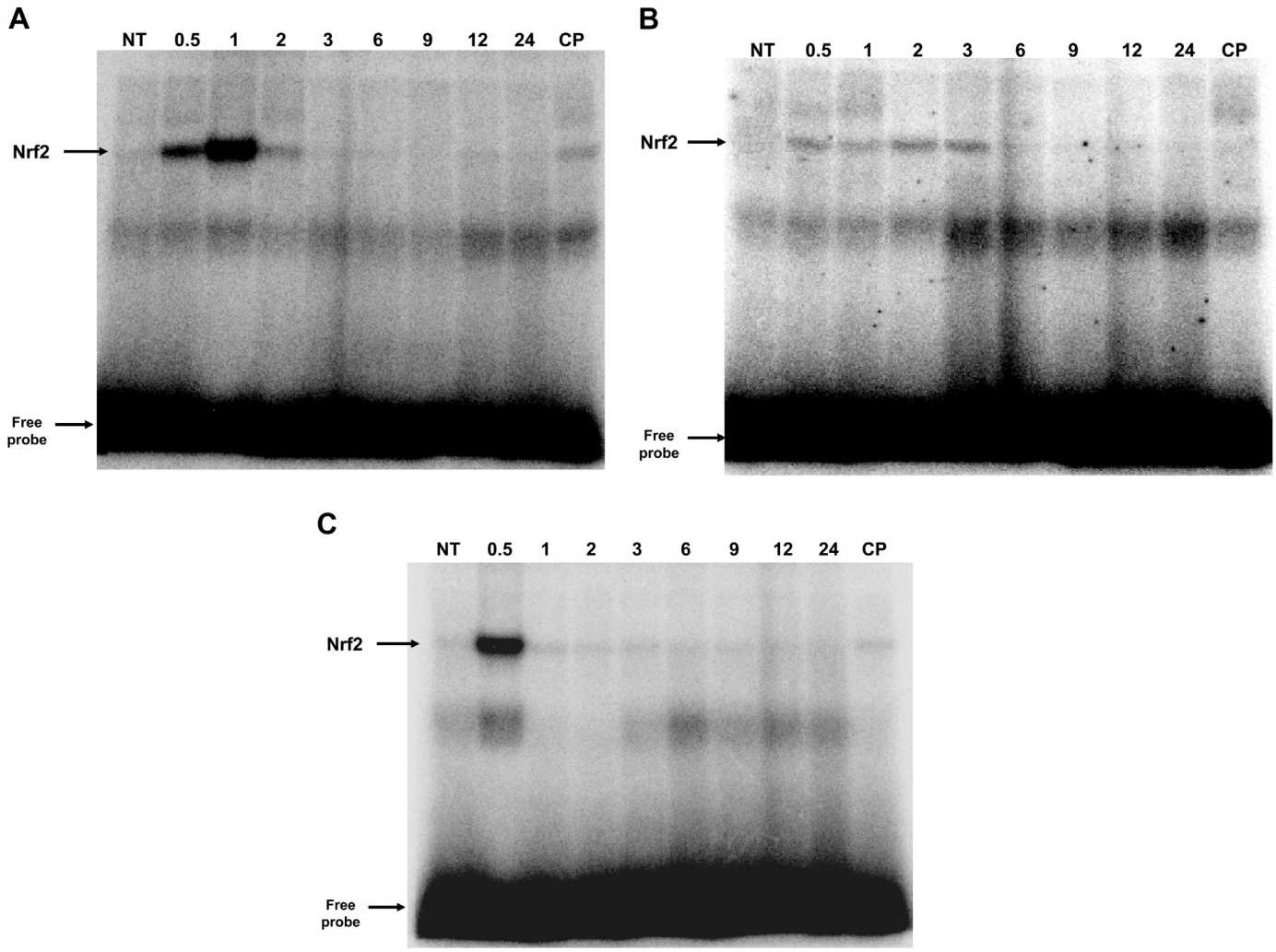

Fig. 3. Nrf2 DNA binding to the ARE sequence was determined by EMSA assay as described in section 2. The images show representative EMSA for: (A) representative EMSA for primary NA treated with tBHQ (50 μM) for 0.5, 1, 2, 3, 6, 9, 12, and 24 hours. (B) Representative EMSA for primary AA treated with tBHQ (25 μM) for 0.5, 1, 2, 3, 6, 9, 12, and 24 hours. (C) Representative EMSA for primary OA treated with tBHQ (25 μM) for 0.5, 1, 2, 3, 6, 9, 12, and 24 hours. NT stands for non-treated cells. The cold probe (CP) was performed using 90% of unlabeled probe and 10% of labeled probe. The figure shows representative gels performed in 3 independent experiments. Abbreviations: ARE, antioxidant response element; EMSA, electrophoretic mobility shift assay; NA, Nrf2, nuclear factor erythroid-derived 2-like 2; tBHQ, tertbuthyl-hydroquinene.

able to activate Nrf2 pathway and protect those cells against a moderate oxidative dare.

4. Discussion

The increase in glial reactivity that has been reported during aging, in combination with an increase in ROS production and a decrease in antioxidant content in the brain (Kanwar and Nehru, 2007) are primary features that trigger the development of neurodegenerative disorders associated with age. Noteworthy, astrocytes have been demonstrated to be susceptible to diverse toxins in particular to MPP+ (Tsai and Lee, 1994, 1998). Previous data from our group (Alarcón-Aguilar et al., 2014) showed that when a pro-oxidant condition induced with MPP+ takes place, astrocytes viability and functionality diminish significantly in direct proportion to the age group tested. Moreover, oxidative damage (lipid peroxidation and protein oxidation) also increased with age. These results may be explained according to Harman free radicals theory of aging (Harman, 2003), which postulates that the decrease in the biochemical and physiological functions associated to aging are because of an increase in accumulated oxidative damage. However, this explanation does not clarify at all one of the controversies still debated in the field: is damage accumulation during aging because of a real increase in ROS production or conversely, is it related to a decrease in antioxidant and repair systems? For example, a decline in antioxidant levels and activity has been reported in the brain of old Fisher rats, especially a drop of γ-GCS, which was accompanied by the expected decrease in GSH (Liu, 2002). In a similar study Zhu et al. (2006) reported a decrease in GSH and γ-GCS associated to an elevation in GSSG and lipid peroxidation, along with an increase in enzymes like GST and GGT, pointing toward a decline in the anti-oxidant system associated to aging.

On the other hand, an increase in superoxide anion and free ion coupled to a higher lipid and protein oxidative damage was observed in astrocytes derived from old animals, which did not present variations in the antioxidant enzymes, thus suggesting that astrocytes from senile animals are more susceptible to oxidative damage (Klamt et al., 2002; Papadopoulos et al., 1998). Other experiments carried out with primary astrocytes obtained from SAMP8 mice (senescent accelerated prone mice), confirmed the increase in ROS production and macromolecular damage (García-Matas et al., 2008). Similar results were recently obtained in

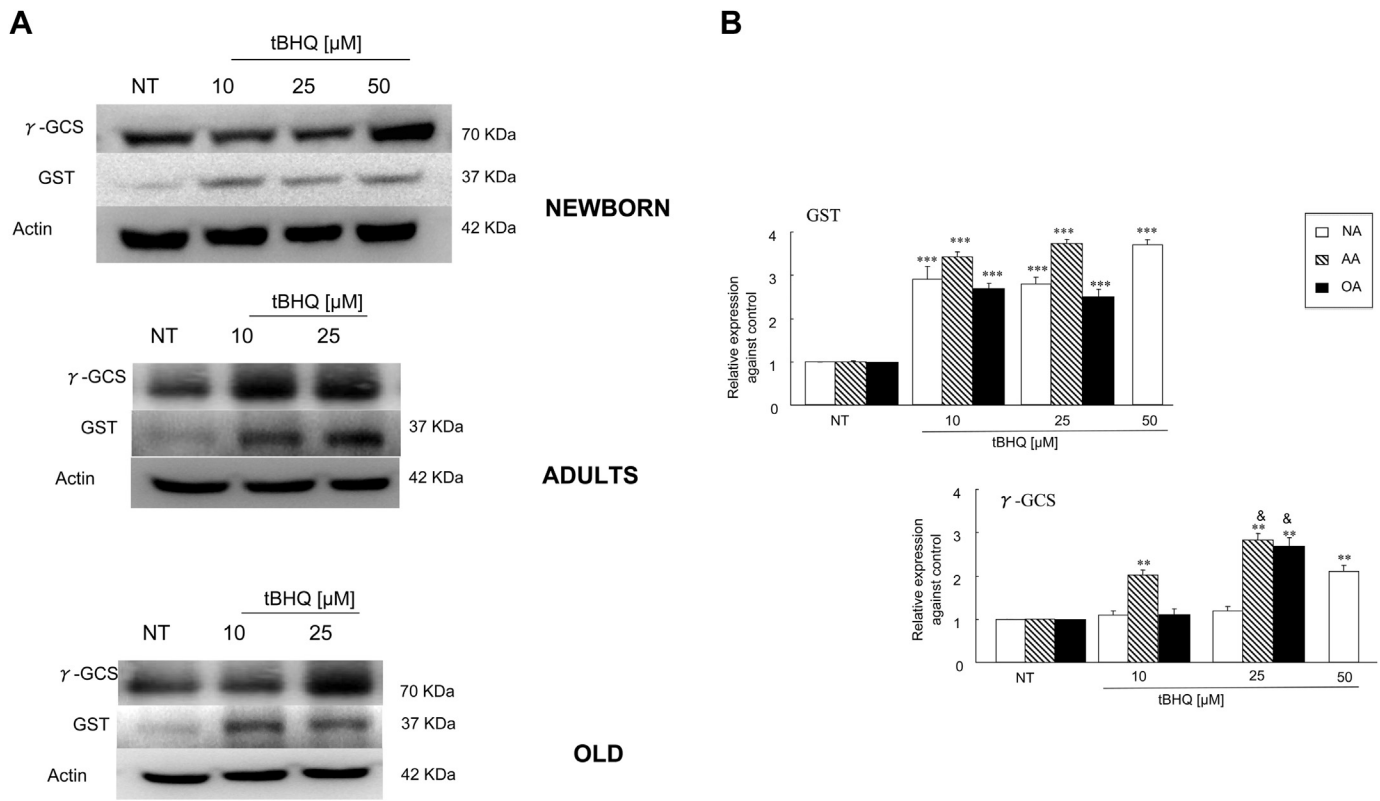

Fig. 4. Antioxidant response in astrocytes pre-treated with tBHQ. (A) Primary astrocytes were pretreated with different concentrations of tBHQ for 24 hours and GST and γGCS were determined by Western blot as described previously. (B) Densitometric analysis was normalized against actin house keeping and then against NA as control, which was considered 100%. Each point represents the mean ± SD of 3 determinations performed in independent experiments. NA (white bars), AA (shredded bars), and OA (black bars). Statistical significance with respect to untreated cells: $^{*}\ p < 0.05$, $^{**}\ p < 0.01$ were considered; that is, treated NA, AA, and OA versus untreated NA. Statistical difference between AA and OA versus NA treated with the same tBHQ concentration was designated as $^{\&}\ p < 0.05$. Abbreviations: SD, standard deviation; tBHQ, tertbuthyl-hydroquinene.

astrocytes derived from newborn (1-day-old), mature (12-month-old), old (25-month-old), and senescent (31-month-old) mice, showing that the antioxidant capability and GSH metabolism was not altered in old and senescent astrocytes, thus proposing that the oxidative stress might be associated to extrinsic factors (Liddell et al., 2010).

There is a vast literature demonstrating that the phenolic compound tBHQ, which has been used as an antioxidant and preservative in the food industry, is a well-known Nrf2 inducer (Kraft et al., 2004; Li et al., 2005), which protects cells against oxidative stress (Li et al., 2005; Yan et al., 2010). Nrf2 is known to activate the cellular antioxidant defense system (Motohashi and Yamamoto, 2004; Osburn et al., 2006). Lee et al. (2003) showed that Nrf2 induction by tBHQ treatment increased γGCS, GST, and NQO1 expressions, which protected primary astrocytes against H_2O_2 and PFG oxidative insults.

Nrf2 is a ubiquitous cytosolic protein that is being continuously degraded during cellular homeostasis; however, in response to an increase in oxidative stress, Nrf2 is released from its repressor to be translocated into the nucleus. Our results agree with most of the reports where Nrf2 translocation occurs during the first 2 hours (Niture et al., 2010), even though we found a differential translocation rate among the diverse age groups. However, our data differ from other studies in 2 aspects: first, Nrf2 translocation is usually accompanied by a decrease in cytosolic Nrf2, which was not the case of our model. Herein, Nrf2 total levels augmented in comparison to untreated astrocytes and along with Nrf2 nuclear translocation, there was still a high Nrf2 signal observed in the cytosol. Second, Nrf2 is believed to leave the nucleus rapidly to be degraded via porteasome (Niture et al., 2010) after performing its role; however, through our findings we demonstrated that in primary astrocytes Nrf2 just binds to the ARE for a short period, albeit it is not released from the nucleus during the 24 hours screened. These results are of major relevance because most Nrf2 studies have been carried out in cell lines and not in primary cultures; therefore, signal transduction in our model might evidence dissimilar mechanisms, maybe even closer to what is happening in the animal. Some possible explanations, which would be worthy to further explore, are related to Nrf2 degradation processes. These events hold numerous regulatory mechanisms, which are still on debate. It has been proposed that some members of Src A kinase sub family, such as Fyn, Src, Yes, and Fgr, negatively regulate Nrf2 nuclear levels by phophorylating Tyr568. This modification allows Nrf2 to be exported to the cytosol for its further degradation (Lee et al., 2007). Nevertheless, it is known that GSK3β, which is sensitive to redox changes, controls Src kinases nuclear localization, so the redox state modifications brought on by tBHQ might as well be interfering with this process, leading to Nrf2 nuclear accumulation. In addition, Akt, which is also activated by redox changes during Nrf2 activation pathway (Luna-López et al., 2013), is known to phosphorylate GSK3β, entailing its inactivation (Niture et al., 2011) and thus, causing Nrf2 nuclear accumulation.

As previously mentioned, the principal degradation process for Nrf2 is mediated by Keap-1/Cul3/Rbx1 in the cytosol and in the nucleus (Niture and Jaiswal, 2009; Niture et al., 2009). Because Nrf2 regulates Keap-1 (Lee et al., 2007) and Cul3/Rbx1 expression levels (Kaspar and Jaiswal, 2010), this system might also be impaired, both in the cytosol and the nucleus. Finally, even if there is an excess of Nrf2 in the cytosol, either because of its de novo synthesis, its

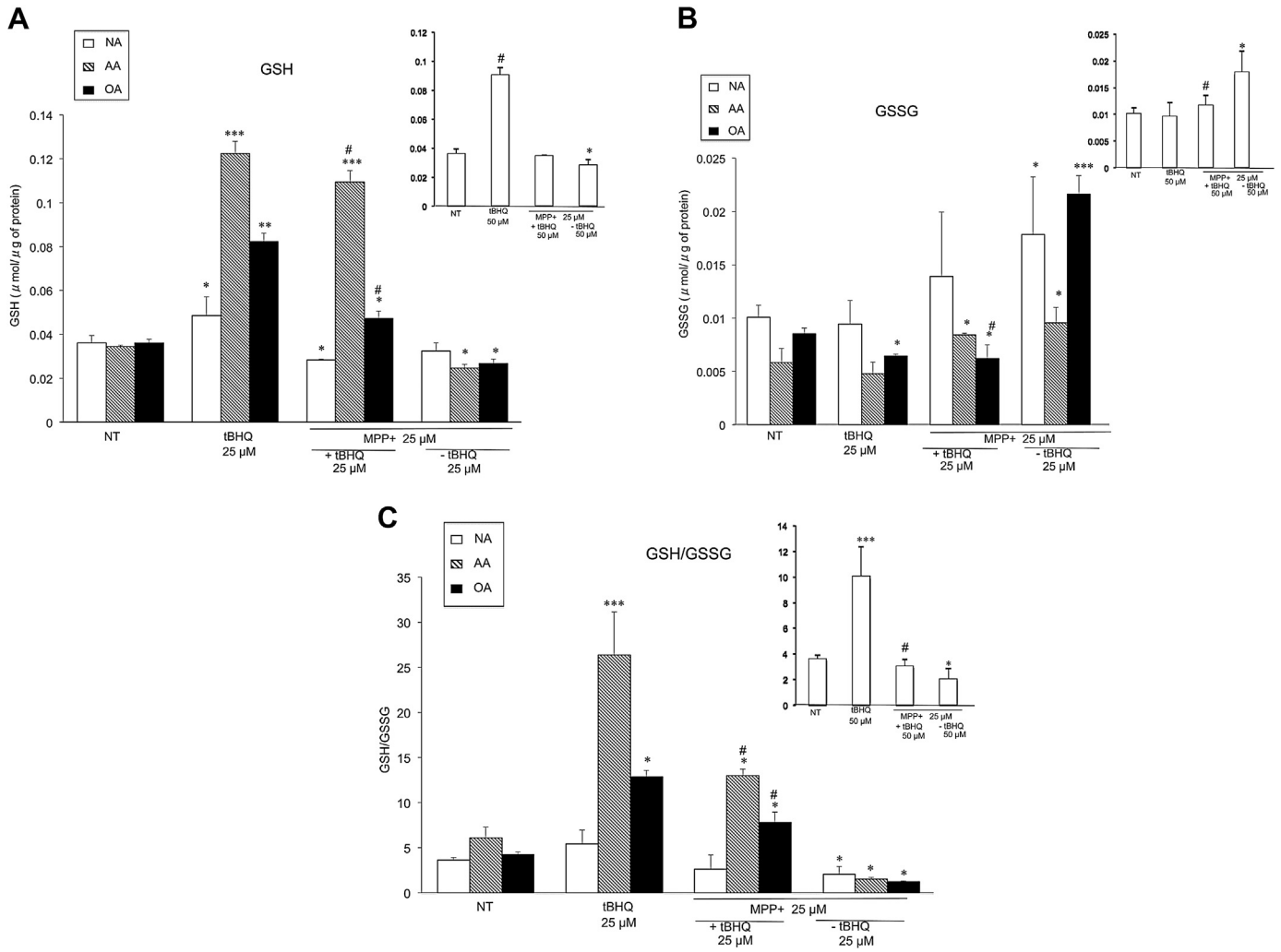

Fig. 5. GSH/GSSG rate. Primary astrocytes were pretreated with 25-μM tBHQ for 24 hours followed by 1-hour MPP+ treatment (25 μM). (A) GSH was determined by fluorometry as described previously and normalized for μg protein. (B) GSSG was determined by fluorometry as described previously and normalized for μg protein. (C) GSH to GSSG ratio. The inset in the figures represent primary NA astrocytes pretreated with 50 μM tBHQ for 24 hours, followed by 1 hour MPP+ treatment (25 μM). NT (non treated), NA (white bars), AA (shredded bars), and OA (black bars). Each bar represents the mean ± SD of 6 determinations performed in 2 independent experiments. Statistical significance with respect to untreated cells or control (NT): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ were considered; statistical difference between tBHQ pretreated and tBHQ untreated astrocytes: # $p < 0.05$. Abbreviations: GSH, glutathione; SD, standard deviation; tBHQ, tertbuthyl-hydroquinene.

impaired degradation, or both, Nrf2 might be incapable to translocate into the nucleus because PKCδ, which phosphorylates Nrf2 in Ser40, could also be compromised (Niture et al., 2009; Zhang and Hannink, 2003).

Another important issue to discuss is why Nrf2 is not binding to the DNA even if it is accumulating in the nucleus. There are at least 2 possible explanations. One is related to Nrf2 competitor Bach1, which is known to translocate into the nucleus in a more delayed event than Nrf2, and compete for the ARE sequence. Bach1 binding to ARE leads to a negative gene expression regulation (Dhakshinamoorthy et al., 2005). The other one is related to the very new findings related to chromatin epigenetic changes and to Nrf2 acetylation, which might favor or obstruct Nrf2-DNA binding. Recent studies have demonstrated that high HDAC enzymes levels, reduce Nrf2-DNA binding (Wang et al., 2012).

An interesting working hypothesis establishes that alterations in antioxidant levels and function in astrocytes during aging might be related to subtle changes in redox homeostasis; hence, in this work, we demonstrated that when astrocytes derived from different age groups are treated with tBHQ, cultured cells are able to increase Nrf2 expression and the corresponding antioxidant response (γ-GCS and GST), as well as GSH levels, thereby activating the mechanisms to survive a toxic MPP+ insult.

tBHQ is known to increase mitochondrial ROS to modulate Nrf2 activity (Imhoff and Hansen, 2010); however, depending on the concentration used, tBHQ was either beneficial or toxic for cells. Indeed, when toxic, tBHQ was noxious up to a point where cell death was observed. Moreover, there was a differential susceptibility to this agent in direct function of age (Fig. 1B), supporting the concept that the changes in the redox status induced by tBHQ may be affordable for some astrocytes (in this case NA) but completely intolerable for others (AA and OA), which in turn might be because of a more prominent and efficient load of antioxidant systems depending on the age.

Consistently with our results, it has been reported that incubation of N27 cells with tBHQ at 30 μM and higher concentrations for 24 hours exert toxic effects (Jakel et al., 2007). tBHQ toxicity has been attributed to the formation of ROS, as well as GSH conjugates and increased CYP1A1 activity (Gharavi et al., 2007).

The literature results, along with our own data, suggest that there might be a most favorable and particular rate to which cells might have a positive response to tBHQ. This could be seen as a threshold

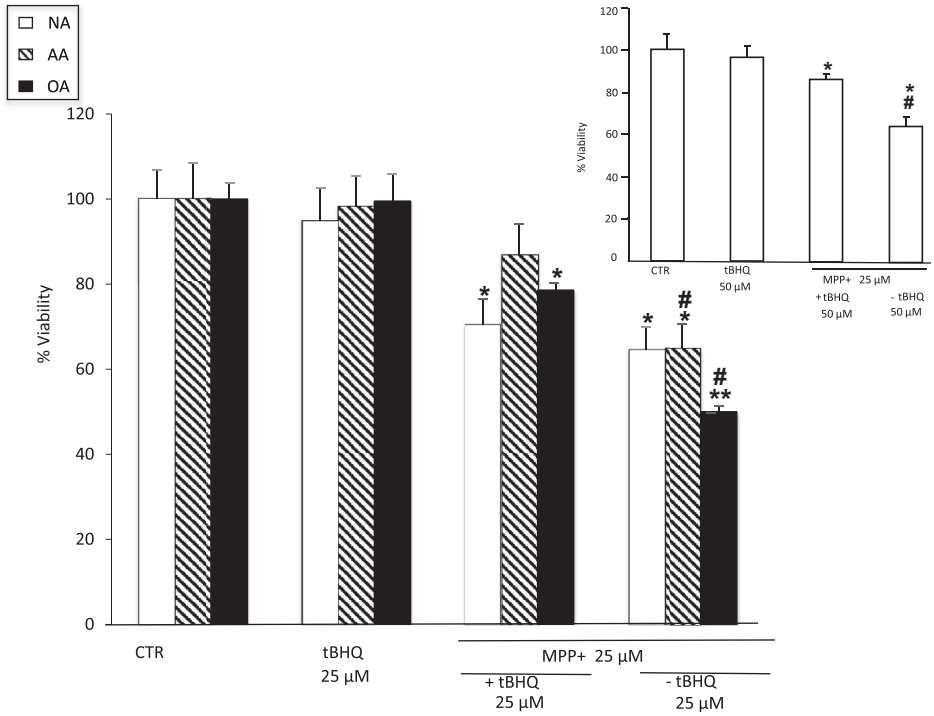

Fig. 6. Cell survival after MPP+ toxicity in astrocytes pretreated with tBHQ Primary astrocytes were pretreated with 25 μM tBHQ for 24 hours, followed by 1 hours MPP+ treatment (25 μM). The inset in the figure represent primary NA astrocytes pretreated with 50-μM tBHQ for 24 hours, followed by 1 hour MPP+ treatment (25 μM). NT (nontreated), NA (white bars), AA (shredded bars), and OA (black bars). Cell viability was assessed by trypan blue as described before. Each bar represents the mean ± SD of 3 determinations performed in independent experiments. Statistical significance * $p < 0.05$, was considered. Statistical significance with respect to untreated or control cells (NT): * $p < 0.05$, ** $p < 0.01$ were considered; statistical difference between tBHQ pretreated and tBHQ untreated astrocytes: # $p < 0.05$. Abbreviations: SD, standard deviation; tBHQ, tertbuthyl-hydroquinene.

that might be dependant on the amount of ROS generated, in contrast to the cellular capacity to modulate oxidative stress and to repair oxidative damage. Moreover, Erlank et al. (2011) have shown that tBHQ in cell growth medium generates H_2O_2 in an equimolar amount to its concentration, which penetrates into the cells, activating Nrf2 signaling. Therefore higher tBHQ concentrations may generate greater H_2O_2 concentrations, which could be cytotoxic. In our case, AA and OA were more susceptible to this effect and did not survive to tBHQ high concentrations. However, it is also known that cells preconditioning with low H_2O_2 concentrations, induces an adaptive response, which activates Nrf2, protecting the cells from further oxidative cytotoxicity (Luna-López et al., 2010). This was probably the scenario for tBHQ lower concentrations in AA and OA, whereas NA required higher tBHQ concentrations to shift their redox state and activate Nrf2, probably because of their antioxidant system proficiency. Furthermore, not only tBHQ or other inductors concentrations are important but as we showed here, the cell type, as well as the age and cellular conditions might be crucial to determine if the same tBHQ concentration can trigger a beneficial or a harmful effect.

The idea of an antioxidant response-threshold is consistent with the physiological and evolutionary processes present in living beings, and in some papers it has been defined as hormesis. Hormesis is a consented terminology that unifies the main mechanism that preconditioning and adaptive responses have in common: the exposure to low levels of stress will activate existing cellular and molecular pathways that will enhance the ability of the cell and organism to withstand to more severe stress (Calabrese, 2008; Mattson, 2008). So, the preconditioning model used in the present work shows that the redox status can be readdress to a more reduced state, as evaluated as the GSH and/or GSSG rate, which correlates with the resistance against MPP+ toxicity. Interestingly, tBHQ preconditioning was successful in protecting AA (45%) and OA (60%) in correlation with the GSH and/or GSSG rate recovery at a low MPP+ concentration (25 μM), but it was not able to recover redox state in OA at a higher dose (50 μM), thereby leading to cell damage (data not shown). These data suggest that cells of old animals might be able to activate the Nrf2 pathway in response to inducers but only in a very narrow concentration range; that is, a tBHQ concentration that is suitable for cell of young animals might harm and even kill cells of old animals. There are, however, still a lot of questions to answer, in particular in regard to the transductional mechanism that induces Nrf2. It is therefore important to understand the oxidative degree and threshold necessary to activate Nrf2 without damaging the cells, as well as its relationship with the repair and cell death initiation.

Most importantly, our results show for the first time that astrocytes derived from adult and aged animals are capable to evoke an antioxidant response, if they are appropriately pretreated with a molecule proficient in modulating redox status. This is of paramount significance while developing therapies related to counteract or protect against age-related pathologies, principally those in which oxidative stress is involved, such as Alzheimer's disease, Parkinson's disease, and other neurodegenerative disorders.

Disclosure statement

The authors have no conflicts of interest to disclose.

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