



Lipopolysaccharide Induces Gliotoxicity in Hippocampal Astrocytes from Aged Rats: Insights About the Glioprotective Roles of Resveratrol

Larissa Daniele Bobermin¹ · Rômulo Rodrigo de Souza Almeida¹ · Fernanda Becker Weber² · Lara Scopel Medeiros² · Lívia Medeiros² · Angela T. S. Wyse^{1,2} · Carlos-Alberto Gonçalves^{1,2} · André Quincozes-Santos^{1,2} 

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Abstract

Astrocytes may undergo a functional remodeling with aging, acquiring a pro-inflammatory state. In line with this, resveratrol represents an interesting strategy for a healthier brain aging since it can improve glial functions. In the present study, we investigated the glioprotective role of resveratrol against lipopolysaccharide (LPS)-induced gliotoxicity in hippocampal aged astrocytes. Astrocyte cultures were obtained from aged rats (365 days old) and challenged *in vitro* with LPS in the presence of resveratrol. Cultured astrocytes from newborn rats were used as an age comparative for evaluating LPS gliotoxicity. In addition, aged rats were submitted to an acute systemic inflammation with LPS. Hippocampal astrocyte cultures were also obtained from these LPS-stimulated aged animals to further investigate the glioprotective effects of resveratrol *in vitro*. Overall, our results show that LPS induced a higher inflammatory response in aged astrocytes, compared to newborn astrocytes. Several inflammatory and gene expression alterations promoted by LPS in aged astrocyte cultures were similar in hippocampal tissue from aged animals submitted to *in vivo* LPS injection, corroborating our *in vitro* findings. Resveratrol, in turn, presented anti-inflammatory effects in aged astrocyte cultures, which were associated with downregulation of p21 and pro-inflammatory cytokines, Toll-like receptors (TLRs), and nuclear factor κ B (NF κ B). Resveratrol also improved astroglial functions. Upregulation of sirtuin 1 (SIRT1), nuclear factor erythroid 2-related factor 2 (Nrf2), and heme oxygenase 1 (HO-1) represent potential molecular mechanisms associated with resveratrol-mediated glioprotection. In summary, our data show that resveratrol can prime aged astrocytes against gliotoxic stimuli, contributing to a healthier brain aging.

Keywords Aging · Astrocytes · Gliotoxicity · Inflammation · Glioprotection · Resveratrol

Introduction

Aging is a complex process characterized by an intrinsic physiological and functional decline of an organism [1, 2], with increased risk for several diseases. In the central nervous system (CNS), cognitive decline is an important feature of aging [3, 4]. Hippocampus is a key structure involved in

learning and memory functions, and represents a vulnerable region for age-related functional alterations [3, 5]. Astrocytes are versatile cells of the CNS that become important candidates for age-related hippocampal alterations, since they display several roles for physiological maintenance of the CNS. They participate in neurotransmitter homeostasis, blood–brain barrier, and neural remodeling [6–8]. In addition, they provide energy metabolites, trophic factors, and antioxidant defenses, including reduced glutathione (GSH), to neurons [6, 9, 10].

Astrocytes also present immune functions since they are able to evoke inflammatory responses in the presence of damage molecules and pathogens [11, 12]. Considering that astrocytes express Toll-like receptor 4 (TLR4), they can be stimulated by the inflammogen lipopolysaccharide (LPS), which leads to nuclear factor κ B (NF κ B) activation and production of several pro-inflammatory cytokines [13, 14]. Moreover, astrocytes from different

✉ André Quincozes-Santos
andrequincozes@ufrgs.br

¹ 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

² Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul—UFRGS, Rua Ramiro Barcelos, 2600 – Anexo Bairro Santa Cecília, Porto Alegre, RS, Brazil

brain areas show an age-dependent functional remodeling [15]. In this context, previous studies from our group have demonstrated that astrocytes acquire a pro-inflammatory profile consistent with the inflammaging process, which is characterized by increased expression and/or release of inflammatory mediators, such as tumor necrosis factor alpha (TNF- α), interleukins 1 β (IL-1 β) and 6 (IL-6), NF κ B p65 subunit, and cyclooxygenase 2 (COX-2) [16–20].

The modulation of inflammaging can represent an interesting strategy for a healthier aging [21–23]. Resveratrol, a natural polyphenolic compound, has received increasingly attention in this research area. In the CNS, the beneficial action of resveratrol can be related to its glioprotective effects [13, 24–27]. Resveratrol is an important activator of sirtuin 1 (SIRT1), which belongs a family of histone and protein deacetylases with several cellular functions [28–31]. SIRT1 can regulate inflammation and aging, whereas decreased SIRT1 levels are correlated with inflammaging and inflammatory-related diseases [28, 32–34]. Besides SIRT1, resveratrol can modulate several signaling molecules, including nuclear factor erythroid 2-related factor 2 (Nrf2), heme oxygenase 1 (HO-1), adenosine receptors, and mitogen activated protein kinase p38 (p38 MAPK) [13, 25–27, 35–37].

The purpose of the present study was to expand the knowledge about the glioprotective potential of resveratrol during aging and inflammatory conditions, by investigating the multi-target effects of resveratrol on LPS-induced gliotoxicity in hippocampal astrocyte cultures obtained from aged rats. For this, different experimental approaches were used: (i) we initially evaluated age-related effects of LPS and resveratrol in primary astrocyte cultures obtained from newborn and aged rats; (ii) after, we exposed astrocyte cultures obtained from aged rats to LPS and resveratrol (in vitro experimental model) to evaluate inflammatory, oxidative, and glial parameters, as well as potential mechanisms associated with resveratrol-mediated glioprotection, particularly SIRT1, Nrf2 and HO-1; (iii) finally, to corroborate our findings related to the in vitro experimental model with LPS, we induced an in vivo acute systemic inflammation in aged rats with LPS and evaluated biochemical, inflammatory, and gene expression parameters in blood serum, cerebrospinal fluid (CSF), and/or hippocampal tissue, as well as astrocyte cultures were obtained from aged rats stimulated in vivo with LPS to further investigate the glioprotective roles of resveratrol in vitro. To our knowledge, the present study provides the first demonstration that resveratrol can act on astrocytes derived from aged rats by improving their function and preventing LPS-induced gliotoxicity.

Materials and Methods

Animals

Male Wistar rats (1–2 and 365 days old) were obtained from the breeding colony of Department of Biochemistry (UFRGS, Porto Alegre, Brazil), and 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 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: 21215).

Hippocampal Primary Astrocyte Cultures

Newborn (1–2 days old) and aged (365 days old) Wistar rats had their hippocampi aseptically dissected from cerebral hemispheres, followed by meninges removal. The tissues were digested in Hank's balanced salt solution (HBSS) containing 0.003% DNase using trypsin (0.05%) as previously described [17]. After mechanical dissociation and centrifugation, the cells were resuspended in DMEM/F12 (Gibco) [10% fetal bovine serum (FBS), 15 mM HEPES, 14.3 mM NaHCO₃, 2.5 μ g/mL amphotericin B, and 0.05 mg/mL gentamicin], plated on 6- or 24-well plates pre-coated with poly-L-lysine at a density of $3\text{--}5 \times 10^5$ cells/cm². Astrocytes were cultured at 37 °C in a 5% CO₂ incubator. After 24 h, the culture medium was exchanged; during the first week, the medium was replaced once every 2 days, and from the second week on, once every 4 days. From the third week on, astrocytes received medium supplemented with 20% FBS until they reached confluence (around at third week for newborn astrocytes and at approximately the fourth week for mature astrocytes), when were used for the experiments.

In vitro LPS Challenge and Resveratrol Treatment

When the primary astrocyte cultures presented the same number of adherent cells in culture plates (confluence), the culture medium was exchanged with DMEM/F12 1% FBS in the presence or absence of resveratrol (10 μ M, Sigma-Aldrich) for 1 h, in accordance with our previous studies [13, 27, 38]. Then, LPS (1 μ g/ml, Sigma-Aldrich) was added to the incubation medium for 24 h [13], and resveratrol was maintained. A higher dose of LPS (10 μ g/ml) was also tested in the first set of experiments to investigate cell viability and integrity. Alternatively, we co-incubated aged astrocytes with HO-1 inhibitor ZnPP IX (10 μ M) [26] or p38 MAPK

inhibitor SB203580 (10 μ M) [39] to test the roles of these pathways in the modulation of specific parameters by resveratrol and/or LPS.

In vivo Intraperitoneal LPS Injection

Aged Wistar rats (365 days old) received an intraperitoneal injection of 250 μ g/kg LPS (20 animals) [40] or phosphate-buffered saline (control condition – sham; 20 animals). After 24 h, the animals were anesthetized with ketamine/xylazine (75 and 10 mg/kg, respectively) and placed in a stereotaxic apparatus for CSF collection from the cisterna magna. Blood samples were obtained by intracardiac puncture. Hippocampi were aseptically dissected from animals and used for hippocampal tissue analyses or preparation of primary astrocyte cultures. Blood, CSF, and hippocampal tissue samples were frozen at -80 °C until further analyses. Primary astrocyte cultures were immediately prepared as described above.

In vitro resveratrol treatment of primary astrocyte cultures derived from LPS-injected rats

At confluence, primary astrocyte cultures obtained from LPS-injected and control rats had their culture medium exchanged with DMEM/F12 1% FBS and were incubated in the presence or absence of resveratrol (10 μ M) for 24 h.

MTT Reduction Assay

MTT (methylthiazolyldiphenyl-tetrazolium bromide, Sigma-Aldrich) was added to the culture medium at a concentration of 50 μ g/mL and cells were incubated for 3 h at 37 °C in an atmosphere of 5% CO₂. Subsequently, the medium was removed and the MTT crystals were dissolved in dimethylsulfoxide. Absorbance values were measured at 560 nm and 650 nm [39]. The results are expressed as percentages relative to the control conditions.

Propidium Iodide Incorporation Assay

Astrocyte cultures were incubated with 7.5 μ M PI for 30 min in 5% CO₂ at 37 °C. The optical density of fluorescent nuclei (labeled with PI), indicative of cell death, was determined with Image J software. Density values obtained are expressed as a percentage of the control value.

Lactate Dehydrogenase (LDH) Assay

The release of the enzyme lactate dehydrogenase (LDH) was assessed measuring its activity in the culture medium (100 μ L) of astrocytes using a commercial UV assay (Bioclin, Brazil). Results are expressed as percentages of the control value.

Actin-Labeling Analysis

For actin-labeling analysis, cells were fixed for 20 min with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), rinsed with PBS, and then permeabilized for 10 min in PBS containing 0.2% Triton X-100. After, an incubation with 10 μ g/mL rhodamine-labeled phalloidin in PBS for 20 min was performed, followed by two washes with PBS. Cell nuclei were stained with 0.2 μ g/mL of 4', 6'-diamidino-2-phenylindole (DAPI) for further 20 min. Astrocyte cultures were analyzed using a Nikon microscope and photographed with a digital camera DXM1200C and TE-FM epi-fluorescence accessory.

Inflammatory Response Measurement

The levels of tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), interleukin-6 (IL-6), interleukin-10 (IL-10), and monocyte chemoattractant protein-1 (MCP-1/CCL2) were measured in the culture medium of astrocytes, serum, CSF, or hippocampal homogenates using ELISA commercial kits. The assay ranges for the kits are the following: 16 to 2000 pg/ml for TNF- α (Invitrogen, catalog #88-7340-22); 31.3 to 2000 pg/ml for IL-1 β (Invitrogen, catalog #BMS630); 31.3 to 2000 pg/ml for IL-6 (Invitrogen, catalog #BMS625); 15.6 to 1000 pg/ml for IL-10 (Invitrogen, catalog #BMS629); and 7.8–500 pg/ml for MCP-1 (Invitrogen, catalog #BMS631INST). The results are expressed in pg/ml for culture medium of astrocytes, serum, and CSF samples. For hippocampal tissue homogenates, the results were calculated as pg/ml and then corrected by the total amount of protein, thus they are expressed as pg/mg protein.

RNA Extraction and Quantitative RT-PCR

Total RNA was isolated from astrocyte cultures and hippocampal tissue using TRIzol Reagent (Invitrogen). The concentration and purity of the RNA were determined spectrophotometrically at a ratio of 260:280. Then, 1 μ g of total RNA was reverse transcribed using Applied Biosystems™ HighCapacity complementary DNA (cDNA) Reverse Transcription Kit (Applied Biosystems) in a 20- μ L reaction according to manufacturer's instructions. The messenger RNA (mRNA) encoding TNF- α (#Rn9999017_m1), IL-1 β (#Rn00580432_m1), TLR2 (#Rn02133647_s1), TLR4 (#Rn00569848_m1), p65 NF κ B (#Rn01399572_m1), COX-2 (#Rn01483828_m1), inducible nitric oxide synthase (iNOS; #Rn00561646_m1), Nrf2 (#Rn00582415_m1), HO-1 (#Rn01536933_m1), glutamate cysteine ligase (GCL; #Rn00689046_m1), SIRT1 (#Rn01428096_m1), p21 (#Rn00589996_m1), glutamate/aspartate transporter (GLAST; #Rn00570130_m1), glutamate transporter-1

(GLT-1; #Rn00691548_m1), glutamine synthetase (GS; #Rn01483107_m1), and β -actin (#Rn00667869_m1) was quantified using the TaqMan real-time RT-PCR system using inventory primers and probes purchased from Applied Biosystems. Quantitative RT-PCR was performed using the Applied Biosystems 7500 Fast system during 40 cycles of amplification. Target mRNA levels were normalized to β -actin levels and expressed relative to the levels in control astrocytes using the $2^{-\Delta\Delta C_t}$ method [41].

p38 MAPK Levels

The p38 MAPK levels were detected using the rat p38 MAPK ELISA commercial kit (Sigma-Aldrich, catalog #PM0100). These levels were evaluated in the cell lysate suspended in a specific buffer from the ELISA kit. The assay range of the ELISA kit is 31.2 to 2000 pg/ml. The results are expressed as percentages relative to the control levels.

NF κ B Transcriptional Activity

Nuclear content was obtained treating lysed cells with 1% Igepal CA-630, followed by a vortex for 10 s and centrifugation at $400\times g$ for 5 min. After centrifugation, nuclei pellets were resuspended in specific buffer (provided by ELISA kit manufacturer) and assayed for p65 NF κ B using a commercial ELISA kit with some modifications (Invitrogen, catalog #85–86,081). The results are expressed as percentages relative to the control levels.

DCFH Oxidation

Intracellular ROS levels were detected using DCFH-DA (Sigma-Aldrich). DCFH-DA was added to the medium at a concentration of 10 μ M and astrocytes 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 with excitation at 485 nm and emission at 520 nm [42]. The results are expressed as percentages relative to the control conditions.

JC-1 Assay

To determine the mitochondrial membrane potential ($\Delta\Psi_m$), cells were incubated for 30 min with JC-1 (Invitrogen, 2 μ g/mL) [43]. Then, cells were washed once with Hank's balanced salt solution (HBSS) and the fluorescence was immediately read using an excitation wavelength of 485 nm and emission wavelengths of 540 and 590 nm. The $\Delta\Psi_m$ was calculated using the ratio of 595 nm (red fluorescent J-aggregates) to 535 nm (green monomers). The results are expressed as percentages relative to the control conditions.

RNA Oxidation

Total RNA was isolated from astrocyte cultures using TRIzol Reagent (Invitrogen). The concentration and purity of RNA were determined spectrophotometrically at a ratio of 260:280. The measurement of RNA oxidation was carried out using a rat ELISA kit from Cayman Chemical specific for 8-hydroxyguanosine. The results are expressed as fold relative to control.

Glutathione Levels

Glutathione (GSH) levels were assessed as previously described [44]. Astrocyte lysate suspended in a sodium phosphate buffer with 140 mM KCl was diluted with a 100 mM sodium phosphate buffer (pH 8.0) containing 5 mM EDTA, and the protein was precipitated with 1.7% meta-phosphoric acid. The supernatant was assayed with o-phthalaldehyde (at a concentration of 1 mg/mL methanol) at 22 °C for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 and 420 nm, respectively. A calibration curve was performed with standard GSH solutions at concentrations ranging from 0 to 500 μ M. The results are expressed in nmol/mg protein.

Glutamate Cysteine Ligase Activity

Glutamate cysteine ligase (GCL) was assayed according to Seelig et al., with slight modifications [45]. Cell lysate, suspended in a sodium phosphate buffer containing 140 mM KCl, was diluted with 100 mM sodium phosphate buffer (pH 8.0) containing 5 mM EDTA. The enzyme activity was determined after monitoring the NADH oxidation at 340 nm in sodium phosphate/KCl (pH 8.0) containing 5 mM ATP- Na_2 , 2 mM phosphoenolpyruvate, 10 mM L-glutamate, 10 mM L- α -amino-butyrate, 20 mM MgCl_2 , 2 mM EDTA- Na_2 , 0.2 mM NADH, and 17 μ g of pyruvate kinase/lactate dehydrogenase. The results are expressed in nmol/mg protein/min.

Glutathione Peroxidase (GPx) Activity

Glutathione peroxidase (GPx) activity was measured using the RANSEL kit from Randox (Autrim, UK). The concentration of GPx in lysed cells is assessed by measuring the absorption of NADPH at 340 nm. The results are expressed as U/mg protein.

Glutamate Uptake

The glutamate uptake assay was performed using H^3 -labelled glutamate [18]. Briefly, astrocytes were rinsed once with PBS and were incubated at 37 °C in HBSS containing the

following components (in mM): 137 NaCl, 5.36 KCl, 1.26 CaCl₂, 0.41 MgSO₄, 0.49 MgCl₂, 0.63 Na₂HPO₄, 0.44 KH₂PO₄, 4.17 NaHCO₃, and 5.6 glucose, adjusted to pH 7.4. Subsequently, 0.1 mM L-glutamate and 0.33 μ Ci/ml L-[3,4-³H] glutamate were added to initiate the assay. After 7 min, the incubation was stopped by removal of the medium and rinsing the cells twice with ice-cold HBSS. The cells were then lysed in a 0.5 M NaOH solution and incorporated radioactivity was measured using a scintillation counter. Sodium-independent uptake was determined using ice-cold N-methyl-D-glucamine instead of sodium chloride. Sodium-dependent glutamate uptake was obtained by subtracting the sodium-independent uptake from the total uptake. The results are expressed as nmol/mg protein/min.

Glutamine Synthetase (GS) Activity

The activity of glutamine synthetase (GS) was determined as previously described [18]. Briefly, the cell homogenate was added to a reaction mixture containing 10 mM MgCl₂, 50 mM L-glutamate, 100 mM imidazole–HCl buffer (pH 7.4), 10 mM 2-mercaptoethanol, and 50 mM hydroxylamine–HCl. The addition of 10 mM ATP started the reaction, which was continued for 15 min at 37 °C. A solution containing 370 mM ferric chloride, 670 mM HCl, and 200 mM trichloroacetic acid was then added to stop the reaction. After centrifugation, the absorbance of the supernatant was measured at 530 nm. A calibration curve was prepared using γ -glutamyl hydroxamate and treated with ferric chloride reagent. The results are expressed in μ mol/mg protein/h.

BDNF and GDNF Measurements

Extracellular levels of BDNF and GDNF were measured in the culture medium of primary astrocyte cultures using ELISA kits. The assay ranges are 12.3 to 3000 pg/ml for BDNF (Invitrogen, catalog #ERBDNF) and 31.2 to 2000 pg/ml for GDNF (Abcam; catalog #ab213901). The results are expressed as percentages relative to the control conditions.

Protein Assay

Protein content was measured using Lowry's method with bovine serum albumin as a standard [46].

Statistical Analyses

Differences among groups were statistically analyzed using one-way or two-way analysis of variance (ANOVA) followed by Tukey's test. Student's *t* test was used for comparisons between control and LPS in vivo injection in aged Wistar rats. Correlations were analyzed by Pearson correlation coefficient. All analyses were performed using the GraphPad

Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA). Values of $P < 0.05$ were considered significant (*a* refers to statistically significant differences from the control; *b* refers to statistically significant differences from LPS challenge). Outliers were removed because some astrocyte cultures did not reach the confluence at the same time.

Results

How Do LPS and Resveratrol Affect Cellular Viability, Integrity, and Morphology of Hippocampal Astrocyte Cultures?

We firstly investigated the potential cytotoxic effect of LPS (1 and 10 μ g/mL) exposure for 24 h on hippocampal astrocyte cultures obtained from newborn and aged (365 days old) rats. Only at 10 μ g/mL, LPS decreased MTT reduction ($P < 0.0001$), indicating an impairment of mitochondrial activity, and consequently, cellular viability (Fig. 1B). In addition, the same dose of LPS caused a loss of membrane integrity that was evaluated by PI incorporation (Fig. 1C; $P < 0.0001$) and extracellular LDH activity (Fig. 1D; $P < 0.0001$) assays. However, both doses of LPS at 24 h did not affect viability and integrity of astrocyte cultures from newborn rats (Fig. 1B–D). Thus, we chose 1 μ g/mL LPS as the dose to challenge our cultured astrocytes.

We also confirmed that 10 μ M resveratrol did not present cytotoxic effects in hippocampal astrocytes derived from newborn and aged rats (Fig. 1B–D). Moreover, resveratrol was able to attenuate the effects of LPS on MTT reduction and extracellular LDH activity in aged astrocyte cultures.

Newborn and aged astrocytes showed significant staining for actin, the major determinant of the cell morphology, with parallel arrangement of stress fiber organization (Fig. 1E). LPS (1 μ g/mL) caused a slight actin reorganization, particularly in aged astrocytes, which did not occur in the presence of resveratrol.

How Do LPS and Resveratrol Modulate the Inflammatory Response and the Senescence Marker p21 in Cultured Hippocampal Astrocytes from Newborn and Aged Animals?

LPS (1 μ g/mL) caused a markedly increase in the release of TNF- α (Fig. 2A; $P < 0.0001$) and IL-1 β (Fig. 2B; $P < 0.0001$) in aged astrocytes. As a comparative, we also exposed newborn astrocyte cultures with LPS 1 μ g/mL and as expected, it was observed an increase in the release of TNF- α and IL-1 β (Fig. 2A, B; $P < 0.0001$ and $P = 0.0014$, respectively), but it was weaker than in aged astrocytes ($P < 0.0001$). Additionally, the mRNA expression of both

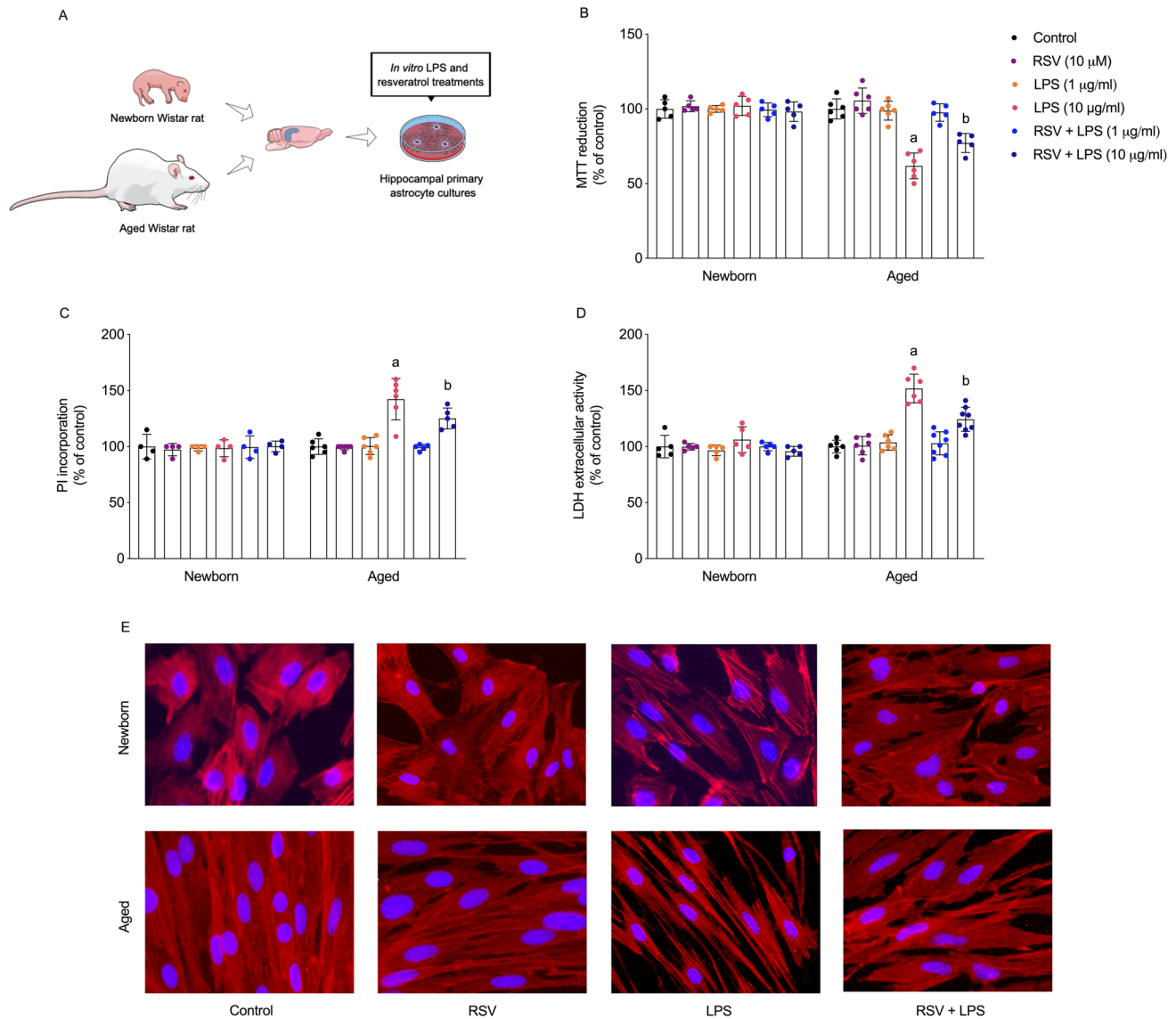


Fig. 1 The effects of LPS and resveratrol on cellular viability, integrity, and actin cytoskeleton of hippocampal astrocyte cultures. Primary hippocampal astrocyte cultures from newborn and aged rats were incubated with LPS (1 or 10 μg/ml) for 24 h in the presence or absence of resveratrol (10 μM), as depicted (A). MTT reduction (B), PI incorporation (C), and LDH extracellular activity (D) were measured. Data are presented as mean ± S.D. and differences among groups were statistically analyzed using two-way analysis of variance (ANOVA), followed by Tukey's test ($n=4-8$ independent cultures

and, at least, duplicate of treatments). Values of $P < 0.05$ were considered significant. *a* refers to statistically significant differences from the control conditions; *b* refers to statistically significant differences from LPS challenge. For actin cytoskeleton analysis (E), cultured astrocytes were incubated with LPS (1 μg/ml) for 24 h in the presence or absence of resveratrol (10 μM). E Representative images of the rhodamine-phalloidin labeling merged with the DAPI staining of newborn and aged astrocyte cultures. RSV, resveratrol

cytokines was upregulated in aged astrocyte cultures (Fig. 2C, D; $P < 0.0001$), but not in newborn astrocytes (Fig. 2C, D). Notably, aged astrocytes showed increased release and expression of TNF-α and IL-1β compared to newborn astrocytes at control conditions.

We then investigated the glial anti-inflammatory effect of resveratrol on newborn and aged astrocytes. Resveratrol prevented LPS-induced inflammation (both expression and release of cytokines), as well as decreased extracellular

levels of TNF-α ($P = 0.0021$) in unstimulated aged astrocytes (Fig. 2C, D).

In addition, we confirmed that aged astrocytes express increased mRNA levels of p21 (90%, $P = 0.0002$), a cellular senescence marker, compared to newborn cultures (Fig. 2E). Moreover, although LPS induced a non-statistically significant increase in p21 mRNA expression in newborn astrocytes (Fig. 2E; $P = 0.0806$), it markedly upregulated p21 in aged astrocytes ($P < 0.0001$), threefold higher than in

In vitro LPS experimental model

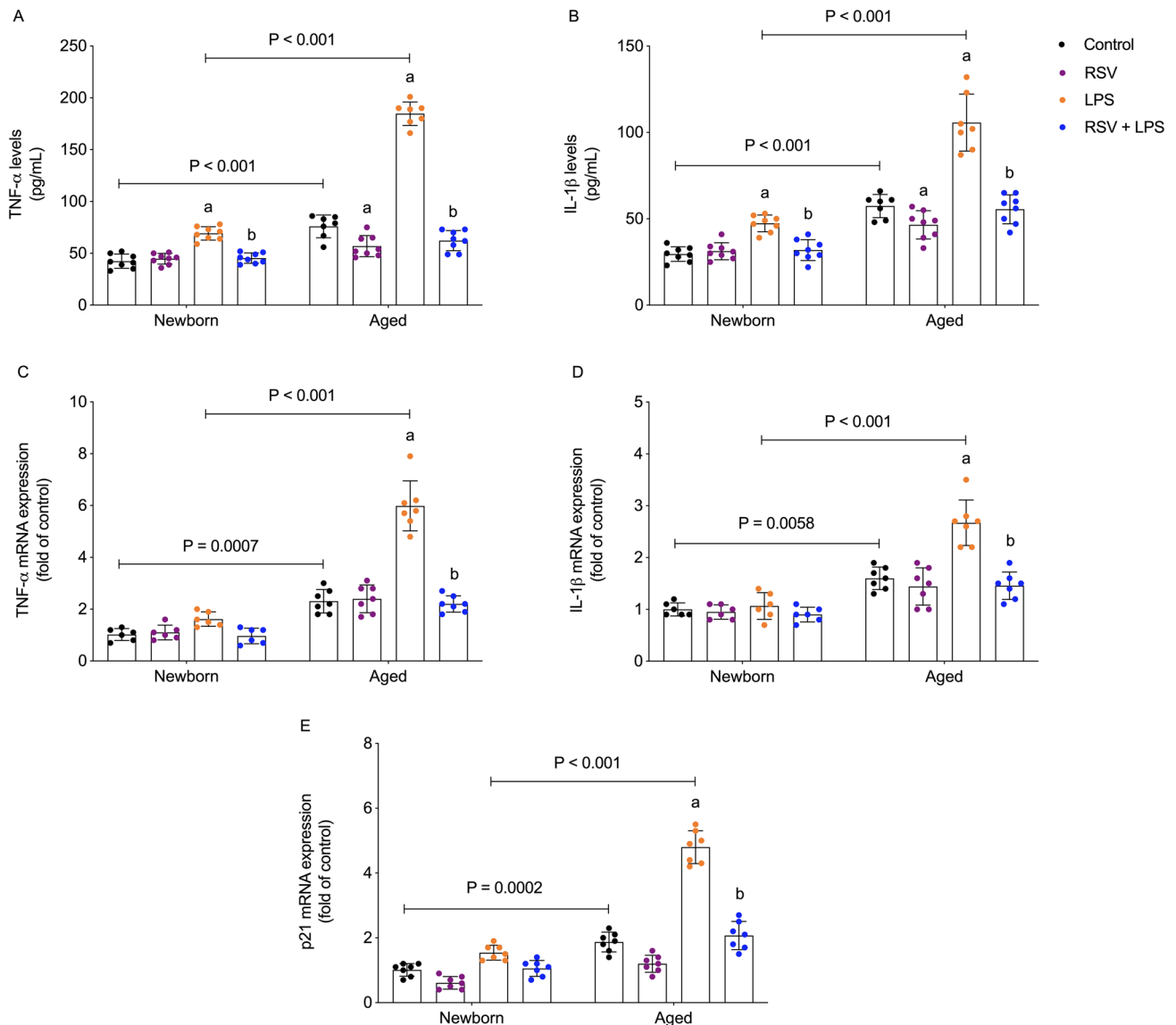


Fig. 2 LPS and resveratrol modulate inflammatory response and senescence marker p21 in cultured hippocampal astrocytes from newborn and aged animals. Primary hippocampal astrocyte cultures from newborn and aged rats were incubated with LPS (1 $\mu\text{g}/\text{ml}$) for 24 h in the presence or absence of resveratrol (10 μM)—experimental design depicted in Fig. 1A. The extracellular levels of TNF- α (A) and IL-1 β (B), and the mRNA expressions of TNF- α (C), IL-1 β (D), and p21 (E) were evaluated. Data are presented as mean \pm S.D. and differences

among groups were statistically analyzed using two-way analysis of variance (ANOVA), followed by Tukey's test ($n=7-8$ independent cultures and, at least, duplicate of treatments). Values of $P < 0.05$ were considered significant. *a* refers to statistically significant differences from the control conditions; *b* refers to statistically significant differences from LPS challenge. The lines indicate differences between ages. RSV, resveratrol

neonatal cultures. Interestingly, resveratrol not only prevented the LPS-induced increase in p21, as maintained its expression similar to newborn levels in unstimulated aged astrocytes (Fig. 2E).

This first set of experiments was performed to ensure that cultured astrocytes obtained from aged (365 days old)

rats represent a model for studying aging, as they present an exacerbation of inflammatory response and senescence marker when compared to newborn cultures. Therefore, to study the glioprotective properties of resveratrol, the subsequent experiments were performed only in hippocampal astrocytes obtained from aged animals.

Resveratrol Exerts Additional Glial Anti-inflammatory Effects on Aged Astrocytes

To further investigate the effect of resveratrol on inflammatory response, we analyzed the extracellular levels of IL-6, MCP-1, and IL-10 released by aged astrocytes. Resveratrol prevented the increase in IL-6 (Fig. 3B) and MCP-1 (Fig. 3C) levels induced by LPS ($P < 0.0001$, LPS versus control conditions). Moreover, resveratrol per se decreased the release of MCP-1 ($P = 0.0371$). However, LPS increased IL-10 release (Fig. 3D; $P < 0.0001$), and resveratrol did not change this effect. Moreover, in unstimulated astrocytes, resveratrol also promoted an increase in IL-10 levels (Fig. 3C; $P = 0.0025$). To investigate the putative role of HO-1 on anti-inflammatory activity of resveratrol, aged astrocytes were co-incubated with ZnPP IX; in the presence of this HO-1 inhibitor, resveratrol did not show its anti-inflammatory effect per se, as well as did not prevent LPS-induced inflammation (Fig. S1).

Resveratrol Modulates Inflammatory Signaling in Aged Astrocytes

We further investigated inflammatory pathways associated with LPS stimulation as potential targets for resveratrol-mediated glioprotection in hippocampal aged astrocytes. The expression of TLR4 (Fig. 4A) and TLR2 (Fig. 4B) was markedly increased by LPS ($P < 0.0001$), and resveratrol prevented this upregulation.

Activation of TLRs is closely related to p38 MAPK/NF κ B signaling pathway [14]. In agreement with that, we found increased levels of p38 MAPK (Fig. 4C) and nuclear p65 NF κ B content (Fig. 4D) in LPS-stimulated astrocytes ($P < 0.0001$). Moreover, LPS also increased mRNA expression of p65 NF κ B (Fig. 4E; $P < 0.0001$). In the presence of resveratrol, however, p38 MAPK- and NF κ B-related pro-inflammatory effects were not observed.

In addition, the mRNA expression of the COX-2, a possible target of NF κ B, (Fig. 4F), was also upregulated in aged astrocytes after LPS exposure ($P < 0.0001$). Resveratrol also prevented this effect.

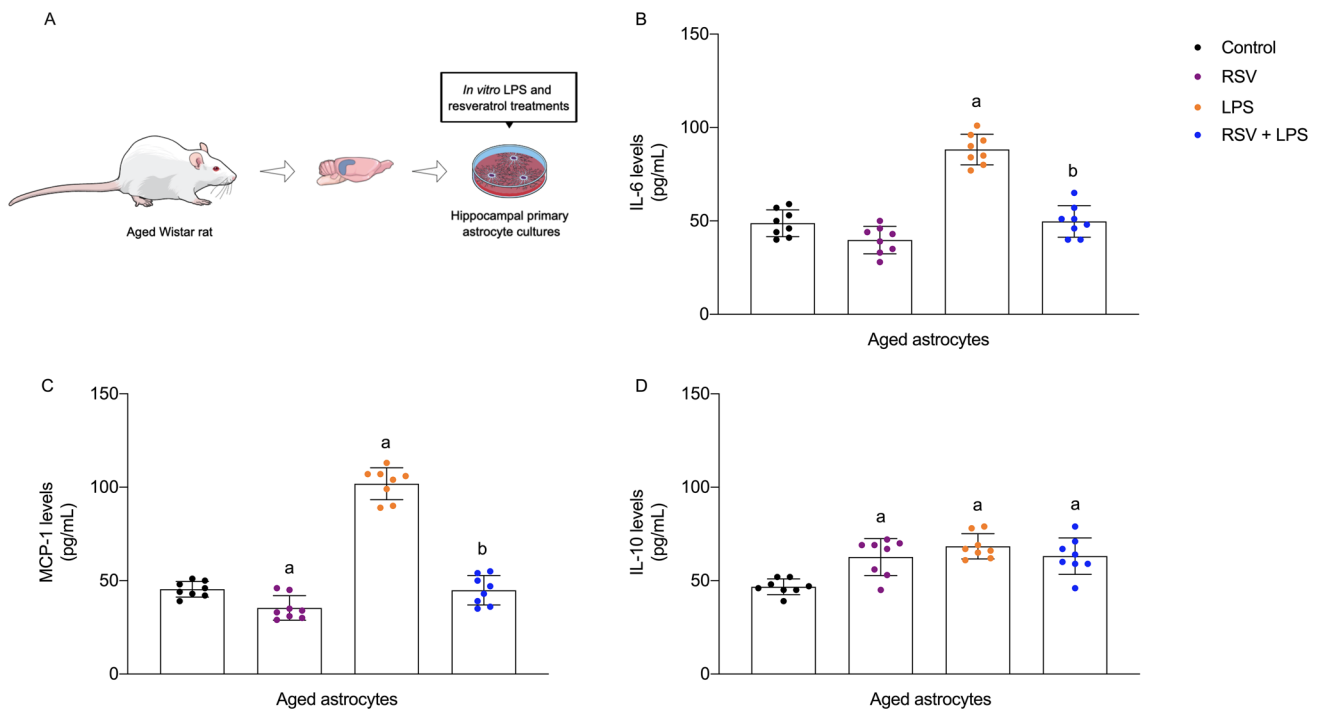


Fig. 3 The effects of LPS and resveratrol on the release of IL-6, MCP-1, and IL-10 in aged astrocytes. Primary hippocampal astrocyte cultures from aged rats were incubated with LPS (1 μ g/ml) for 24 h in the presence or absence of resveratrol (10 μ M), as depicted in (A). The extracellular levels of IL-6 (B), MCP-1 (C), and IL-10 (D) were evaluated. Data are presented as mean \pm S.D. and differences among

groups were statistically analyzed using one-way analysis of variance (ANOVA), followed by Tukey's test ($n = 8$ independent cultures and, at least, duplicate of treatments). Values of $P < 0.05$ were considered significant. *a* refers to statistically significant differences from the control conditions; *b* refers to statistically significant differences from LPS challenge. RSV, resveratrol

In vitro LPS experimental model

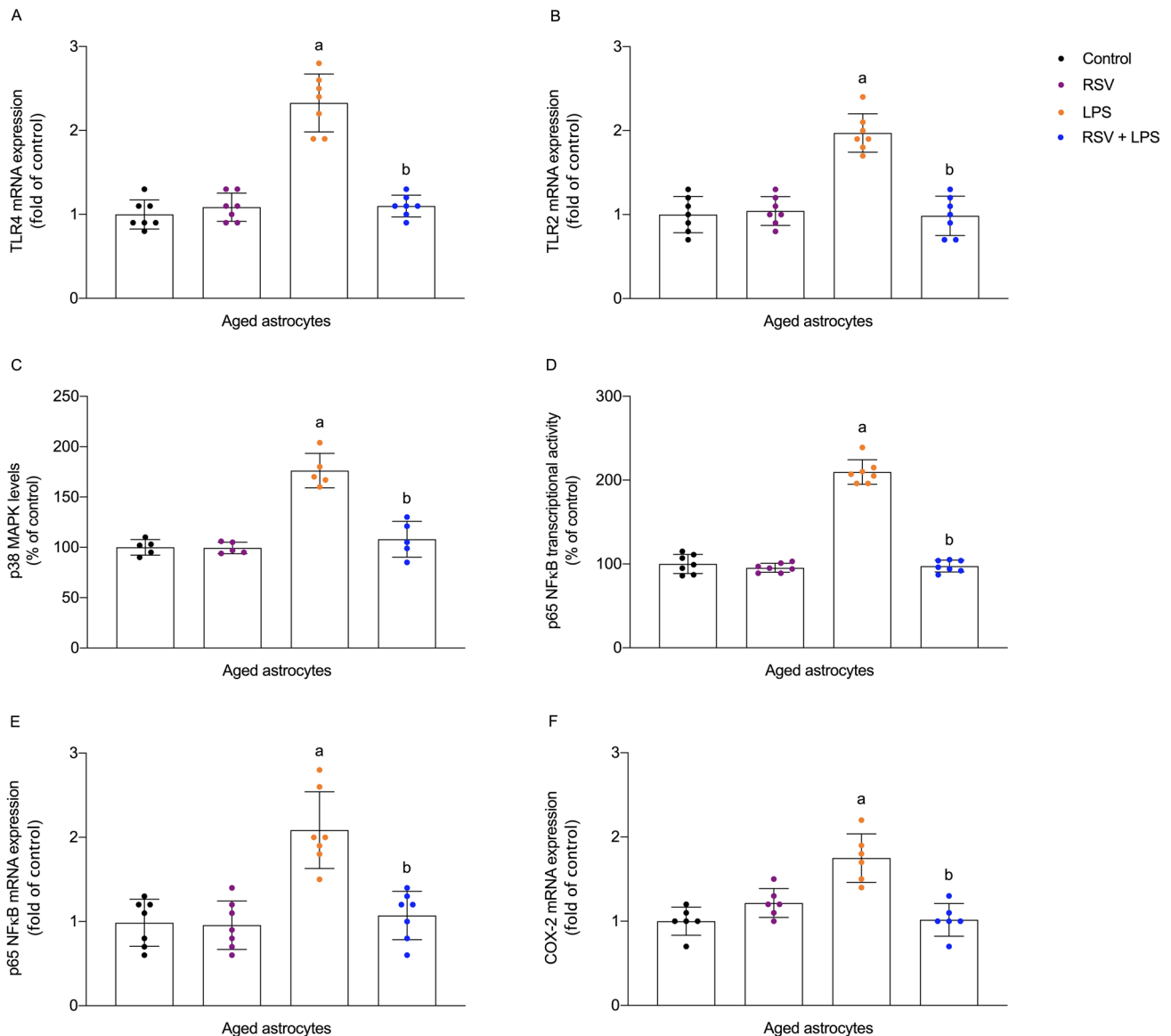


Fig. 4 Resveratrol modulates LPS-induced inflammatory signaling in aged astrocytes. Primary hippocampal astrocyte cultures from aged rats were incubated with LPS (1 $\mu\text{g}/\text{ml}$) for 24 h in the presence or absence of resveratrol (10 μM) – experimental design depicted in Fig. 3A. mRNA expressions of TLR4 (A) and TLR2 (B), and levels of p38 MAPK (C), nuclear content of p65 NF κ B (D), mRNA expressions of p65 NF κ B (E), and COX-2 (F) were evaluated. Data are pre-

sented as mean \pm S.D. and differences among groups were statistically analyzed using one-way analysis of variance (ANOVA), followed by Tukey's test ($n=5-7$ independent cultures and, at least, duplicate of treatments). Values of $P < 0.05$ were considered significant. *a* refers to statistically significant differences from the control conditions; *b* refers to statistically significant differences from LPS challenge. RSV, resveratrol

Resveratrol Prevents LPS-Induced Redox Imbalance in Aged Astrocytes

Although the mRNA levels of iNOS (Table 1) were not affected neither by LPS or resveratrol, LPS increased DCFH oxidation (Table 1; $P < 0.0001$), which was used to evaluate ROS/RNS production. Resveratrol did not present an antioxidant activity per se, but it partially

prevented LPS-induced increase in DCFH oxidation. Alterations in mitochondrial function can be associated with redox imbalance; however, we did not observe any change in the $\Delta\Psi_m$ evaluated by the JC-1 assay (Table 1).

Moreover, inflammation and imbalance in ROS/RNS levels can induce damage to biomolecules. Here, we found that RNA oxidation was markedly increased

Table 1 The effects of resveratrol and LPS on oxidative parameters, glutamate metabolism and trophic factor release in aged hippocampal astrocytes

Parameter	Control (A)	RSV (B)	LPS (C)	RSV + LPS (D)	F	P	Post hoc
Oxidative parameters							
iNOS (mRNA)	1.0 ± 0.21	1.1 ± 0.29	1.1 ± 0.29	1.1 ± 0.22	0.3223	0.8092	
DCFH (%)	100 ± 7.04	101 ± 9.13	203 ± 25.38	165 ± 14.61	51.68	< 0.0001	C ≠ A; D ≠ C; D ≠ A
ΔΨm (%)	100 ± 7.97	98 ± 6.42	102 ± 5.98	95 ± 16.08	0.5242	0.6718	
RNA oxidation (%)	100 ± 8.74	95 ± 6.39	178 ± 15.74	96 ± 7.92	111.1	< 0.0001	C ≠ A; D ≠ C
GSH (nmol/mg prot)	15.33 ± 3.01	25.33 ± 4.88	10.83 ± 3.06	19.83 ± 2.32	19.38	< 0.0001	B ≠ A; D ≠ C
GCL activity (nmol/mg prot/min)	2.82 ± 0.23	3.28 ± 0.48	1.73 ± 0.29	2.95 ± 0.59	14.99	< 0.0001	C ≠ A; D ≠ C
GCL (mRNA)	1.0 ± 0.13	1.3 ± 0.12	0.7 ± 0.12	1.0 ± 0.23	15.13	< 0.0001	B ≠ A; C ≠ A; D ≠ C
GPx activity (U/mg protein)	1.81 ± 0.19	1.85 ± 0.24	2.37 ± 0.39	1.80 ± 0.36	4.766	0.0115	C ≠ A; D ≠ C
Glutamate metabolism							
Glu uptake (nmol/mg prot/min)	0.85 ± 0.19	1.30 ± 0.24	0.48 ± 0.12	0.95 ± 0.19	19.39	< 0.0001	B ≠ A; C ≠ A; D ≠ C
GLAST (mRNA)	1.0 ± 0.20	1.0 ± 0.16	0.5 ± 0.23	0.5 ± 0.15	13.82	< 0.0001	C ≠ A
GLT-1 (mRNA)	1.0 ± 0.15	1.4 ± 0.18	0.4 ± 0.10	1.0 ± 0.10	44.43	< 0.0001	B ≠ A; C ≠ A; D ≠ C
GS activity (μmol/mg prot/h)	1.2 ± 0.22	1.5 ± 0.27	0.7 ± 0.16	1.1 ± 0.26	11.09	0.0003	C ≠ A; D ≠ C
GS (mRNA)	1.0 ± 0.12	1.3 ± 0.11	0.6 ± 0.14	1.0 ± 0.21	28.43	< 0.0001	B ≠ A; C ≠ A; D ≠ C
Trophic factors							
BDNF (%)	100 ± 7.82	137 ± 19.78	62 ± 8.36	115 ± 10.82	50.22	< 0.0001	B ≠ A; C ≠ A; D ≠ C
GDNF (%)	100 ± 6.68	133 ± 8.66	51 ± 12.34	122 ± 14.19	86.70	< 0.0001	B ≠ A; C ≠ A; D ≠ C; D ≠ A

Primary hippocampal astrocyte cultures from aged rats were incubated with LPS (1 μg/ml) for 24 h in the presence or absence of resveratrol (10 μM), and oxidative parameters, glutamate metabolism, and trophic factor release were evaluated, as described in the “Materials and Methods” section. Data are expressed as fold increase for mRNA levels; percentages of control for DCFH oxidation, ΔΨm (JC-1 assay), RNA oxidation, and extracellular content of BDNF and GDNF; nmol/mg protein for GSH levels; nmol/mg protein/min for GCL activity and glutamate uptake; μmol/mg protein/h for GS activity; U/mg protein for GPx. Data are presented as mean ± S.D. and differences among groups were statistically analyzed using one-way analysis of variance (ANOVA), followed by Tukey’s test ($n=6-8$ independent cultures and, at least, duplicate of treatments). Values of $P < 0.05$ were considered significant. F and P values for ANOVA are indicated in the Table. Treatment groups (A, B, C, D) that differ significantly are listed in the post hoc column

in astrocytes challenged with LPS (Table 1; $P < 0.0001$), and resveratrol totally prevented this effect.

With regard to antioxidant defenses, LPS had no effect on GSH levels (Table 1), but resveratrol per se improved the astrocytic content of GSH ($P = 0.0004$), in addition to maintain higher GSH levels in LPS-stimulated astrocytes ($P = 0.0011$). The activity of GCL, a key enzyme in GSH biosynthesis, was impaired by LPS challenge (Table 1; $P = 0.0014$), while resveratrol was able to prevent this effect. Similarly, LPS decreased mRNA expression of GCL (Table 1; $P = 0.0099$), but not in the presence of resveratrol. In unstimulated astrocytes, resveratrol upregulated mRNA levels of GCL ($P = 0.0225$), but this result was not accompanied by a significant increase in the enzymatic activity.

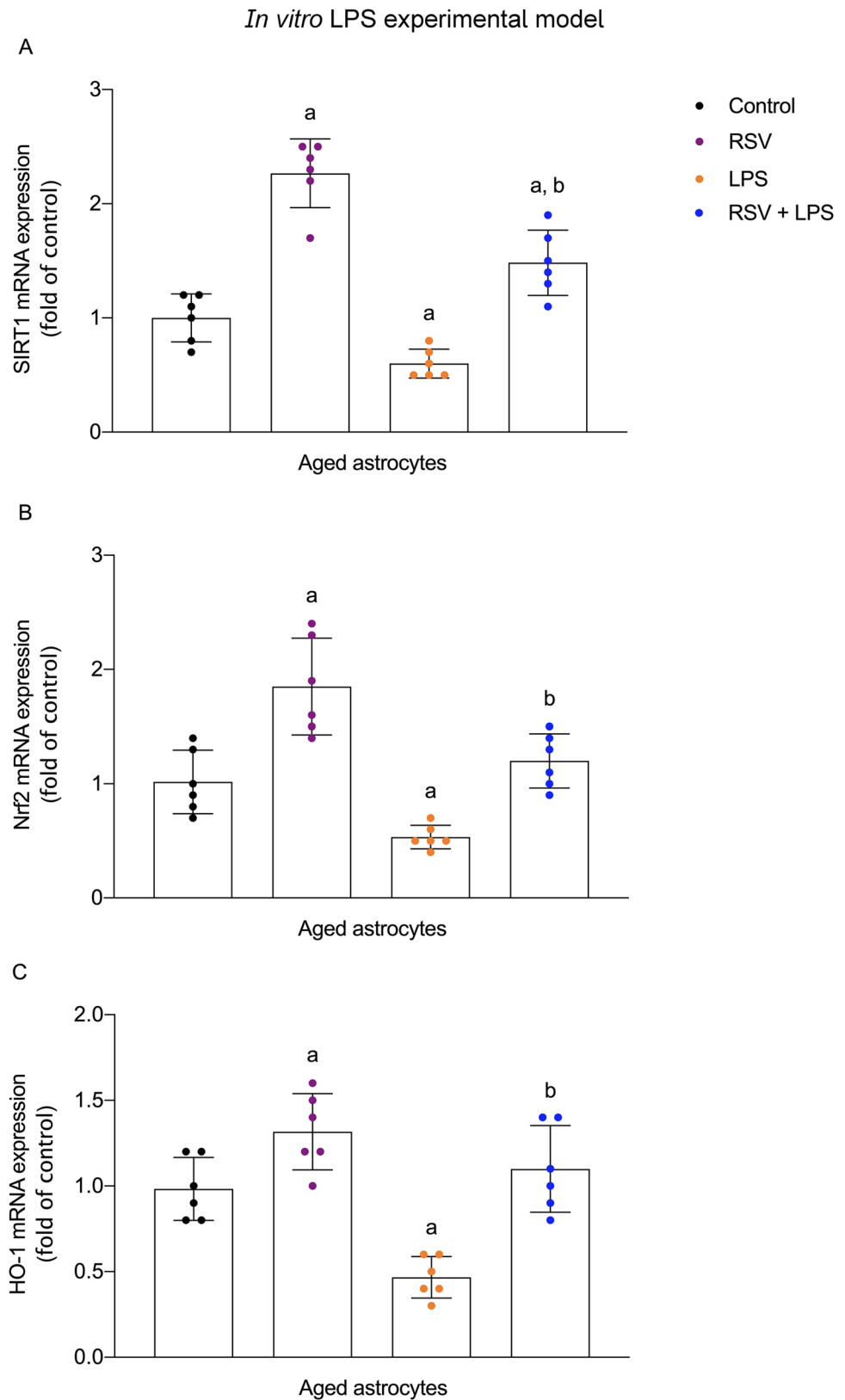
GSH also participates in the enzymatic reaction of GPx. Cultured aged astrocytes challenged with LPS presented a higher activity of GPx (Table 1; $P = 0.0261$). Resveratrol, in turn, re-established GPx activity to control levels.

Resveratrol and LPS Modulate Glutamate Metabolism in Aged Astrocytes

Afterwards, we investigated the effects of LPS and resveratrol on glutamate metabolism. Table 1 displays that LPS decreased glutamate uptake ($P = 0.0139$) and that resveratrol prevented this effect. Moreover, resveratrol per se was able to increase glutamate uptake ($P = 0.0024$). Alternatively, we co-incubated aged astrocytes with HO-1 inhibitor ZnPP IX or p38 MAPK inhibitor SB203580 to test the roles of these pathways in the modulation of glutamate uptake. Inhibition of HO-1 abolished the effect of resveratrol (Fig. S2A), while the inhibition of p38 MAPK affects the action of LPS on glutamate uptake (Fig. S2B).

LPS also induced a decrease in mRNA levels of glutamate transporters GLAST (50%, $P = 0.0009$) and GLT-1 (55%, $P < 0.0001$). Resveratrol prevented only the effects on GLT-1 expression, as well as increased per se the mRNA levels of GLT-1 (Table 1; $P = 0.0007$). Moreover, we found

Fig. 5 SIRT1, Nrf2, and HO-1 signaling pathways are associated with resveratrol-mediated glioprotection. Primary hippocampal astrocyte cultures from aged rats were incubated with LPS (1 μ g/ml) for 24 h in the presence or absence of resveratrol (10 μ M) – experimental design depicted in Fig. 3A. The mRNA expressions of SIRT1 (A), Nrf2 (B), and HO-1 (C) were evaluated. Data are presented as mean \pm S.D. and differences among groups were statistically analyzed using one-way analysis of variance (ANOVA), followed by Tukey's test ($n=6$ independent cultures and, at least, duplicate of treatments). Values of $P<0.05$ were considered significant. *a* refers to statistically significant differences from the control conditions; *b* refers to statistically significant differences from LPS challenge. RSV, resveratrol



that glutamate uptake was positively correlated with the mRNA expression of both GLAST ($r=0.4714$ and $P=0.02$;

data not shown) and GLT-1 ($r=0.7724$ and $P<0.0001$; data not shown).

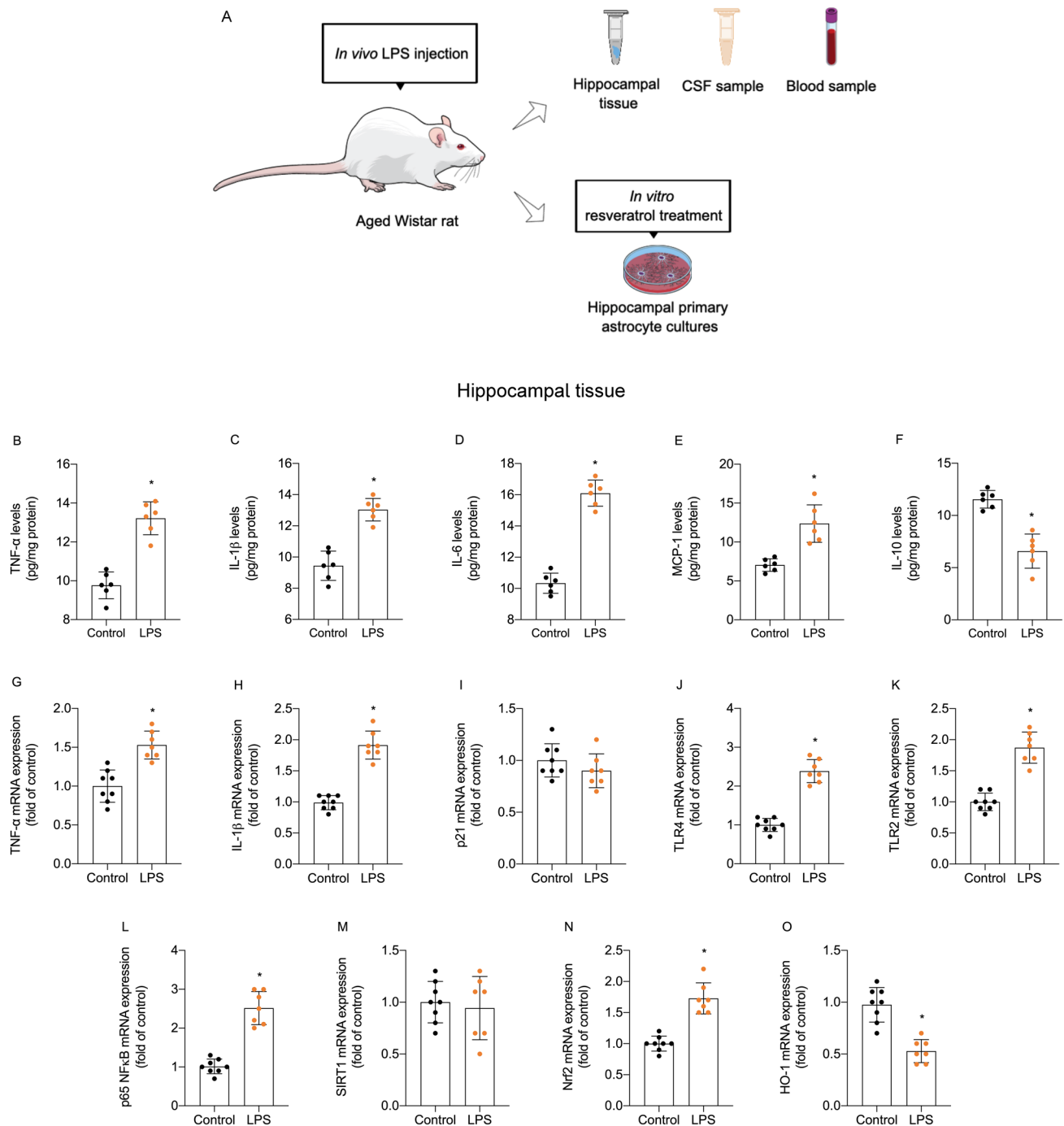


Fig. 6 In vivo injection of LPS changes gene expression in hippocampal tissue of aged rats. Aged (365 days old) Wistar rats were intraperitoneally injected with LPS or saline (control). **A** How blood serum, CSF, and hippocampal tissue were collected after 24 h for further analyses, and astrocyte cultures were prepared. The levels of TNF- α (**B**), IL-1 β (**C**), IL-6 (**D**), MCP-1 (**E**), and IL-10 (**F**), and the mRNA expressions of TNF- α (**G**), IL-1 β (**H**), p21 (**I**), TLR4 (**J**),

TLR2 (**K**), p65 NF κ B (**L**), SIRT1 (**M**), Nrf2 (**N**), and HO-1 (**O**) were evaluated in hippocampal tissue. Data are presented as mean \pm S.D. and differences between control and LPS groups were statistically analyzed using Student's *t* test ($n=5-8$ animals per group and, at least, duplicate of samples). Values of $P < 0.05$ were considered significant. The asterisk indicates statistically significant differences. RSV, resveratrol

After taken up by astrocytes, glutamate might be converted into glutamine by the enzyme GS. In LPS-stimulated astrocytes (Table 1; $P=0.0170$ and $P=0.0001$,

respectively), GS activity and GS expression were decreased, and resveratrol totally prevented these effects. Resveratrol also increased mRNA levels of GS ($P=0.0017$). GS activity

and expression were also positively correlated ($r=0.8600$ and $P<0.0001$; data not shown).

Resveratrol Prevents LPS-Induced Decrease in Trophic Factor Release

We also evaluated the release of BDNF and GDNF (Table 1) after LPS challenge. LPS decreased the levels of BDNF ($P<0.0001$) and GDNF ($P<0.0001$). Resveratrol prevented both effects induced by LPS on trophic factors. Moreover, resveratrol per se increased the release of BDNF and GDNF ($P<0.0001$).

Putative Signaling Pathways Associated with Resveratrol-Mediated Glioprotection Against LPS-Induced Gliotoxicity in Aged Astrocytes

SIRT1, Nrf2, and HO-1 are important mechanisms involved in the glioprotective effects of resveratrol [13, 31]. Aged astrocytes had mRNA levels of SIRT1 (Fig. 5A), Nrf2 (Fig. 5B), and HO-1 (Fig. 5C) decreased after LPS challenge ($P=0.0428$ for SIRT1; $P=0.037$ for Nrf2; $P=0.0013$ for HO-1). Besides to totally prevent these effects, resveratrol maintained SIRT1 expression higher than control levels in LPS-stimulated astrocytes ($P=0.0118$). Importantly, resveratrol significantly upregulated mRNA expression of SIRT1 ($P<0.0001$), Nrf2 ($P=0.0003$), and HO-1 ($P=0.0433$) in unstimulated aged astrocytes. This data regarding HO-1 expression is in accordance with the results incubating aged astrocytes with HO-1 inhibitor, reinforcing the role of Nrf2/HO-1 in resveratrol-mediated glioprotection.

LPS Challenge In vivo Induces Peripheral and Central Inflammatory Responses and Alters Gene Expression in Hippocampus

To confirm that our in vitro experimental model of inflammation may reproduce the main observations of an

in vivo acute systemic inflammation, we challenged aged rats (365 days old) with LPS (intraperitoneal) and evaluated biochemical, inflammatory, and glial parameters in blood serum, CSF, or hippocampal tissue 24 h after LPS administration (Fig. 6A). LPS induced an increase in the levels of TNF- α , IL-1 β , IL-6, and MCP-1, but decreased IL-10, in all samples analyzed (Table 2 and Fig. 6B–F). The biochemical parameters such as glucose, cholesterol, and triglycerides were measured in the blood and were not altered by LPS administration (data not shown). Additionally, we found that LPS increased the mRNA levels of TNF- α ($P<0.0001$; Fig. 6G), IL-1 β ($P<0.0001$; Fig. 6H), TLR4 ($P<0.0001$; Fig. 6J); TLR2 ($P<0.0001$; Fig. 6K), p65 NF κ B ($P<0.0001$; Fig. 6L), and Nrf2 ($P<0.0001$; Fig. 6N), and decreased HO-1 ($P<0.0001$; Fig. 6O) in hippocampal tissue. The expression of SIRT1 (Fig. 6M) and p21 (Fig. 6I) did not change in hippocampus after acute systemic inflammation.

Glioprotective Effects of Resveratrol In vitro After LPS Challenge In vivo in Aged Astrocytes

To further characterize the glioprotective role of resveratrol, we prepared astrocyte cultures from these aged rats stimulated in vivo with LPS. After cellular confluence, we treated these cells with 10 μ M resveratrol for 24 h. It is important to note that we did not observe any change in cellular viability (data not shown). To determine the glial anti-inflammatory effect of resveratrol, we then evaluated the levels of TNF- α , IL-1 β , IL-6, MCP-1, and IL-10, as well as the mRNA expression of TNF- α and IL-1 β . We observed an enhanced inflammatory response in astrocytes derived from LPS-stimulated rats compared to those obtained from control rats, as indicated by the increase in extracellular content and mRNA levels of TNF- α ($P<0.0001$ and $P=0.0003$; Fig. 7A and B, respectively) and IL-1 β ($P<0.0001$ and $P=0.0005$; Fig. 7C and D, respectively). In addition, the release of IL-6 ($P<0.0001$; Fig. 7E) and

Table 2 Inflammatory parameters in blood serum and CSF of LPS-injected aged animals

Parameter	Blood serum		CSF	
	Control	LPS	Control	LPS
TNF- α	54.20 \pm 3.61	77.67 \pm 6.47*	12.22 \pm 1.59	19.16 \pm 1.01*
IL-1 β	59.20 \pm 3.61	99.00 \pm 6.36*	9.70 \pm 0.65	14.20 \pm 1.43*
IL-6	52.20 \pm 9.20	105.50 \pm 9.78*	10.00 \pm 0.79	13.74 \pm 0.79*
MCP-1	46.20 \pm 4.87	87.50 \pm 5.47*	11.50 \pm 0.89	17.14 \pm 1.89*
IL-10	53.60 \pm 4.39	31.17 \pm 6.24*	10.88 \pm 0.95	7.18 \pm 1.17*

Aged (365 days old) Wistar rats were intraperitoneally injected with LPS or saline (control). Inflammatory parameters were evaluated in blood serum and CSF 24 h after injection. The levels of TNF- α , IL-1 β , IL-6, MCP-1, and IL-10 are expressed as pg/ml for blood serum and CSF. Data are presented as mean \pm S.D. and differences between control and LPS groups were statistically analyzed using Student's *t* test ($n=6$ animals per group and, at least, duplicate of samples). Values of $P<0.05$ were considered significant. The asterisks indicate statistically significant differences

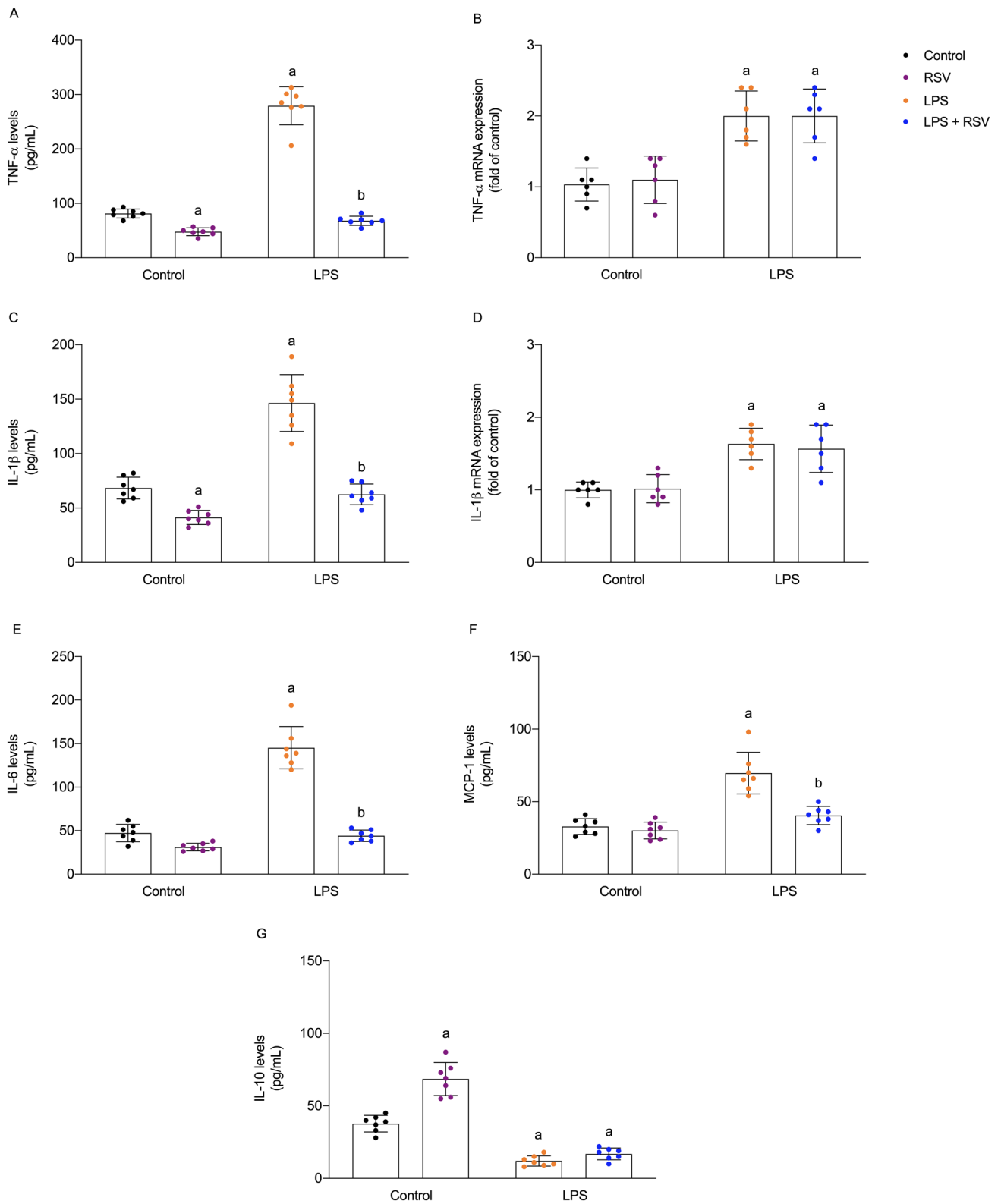
In vivo LPS experimental model

Fig. 7 Glioprotective effects of resveratrol in vitro after LPS challenge in vivo in aged rats. Aged (365 days old) Wistar rats were intraperitoneally injected with LPS or saline (control). After 24 h, astrocyte cultures were prepared and treated with resveratrol (10 μ M; 24 h) at confluence, according to the experimental design depicted in Fig. 6A. The extracellular levels of TNF- α (A), IL-1 β (C), IL-6 (E), MCP-1 (F), and IL-10 (G), and the mRNA expressions of TNF- α (B) and IL-1 β (D) were evaluated. Data are presented as mean \pm S.D. and differences among groups were statistically analyzed using two-way analysis of variance (ANOVA), followed by Tukey's test ($n=6-7$ independent cultures and, at least, duplicate of treatments). Values of $P<0.05$ were considered significant. *a* refers to statistically significant differences from the control conditions; *b* refers to statistically significant differences from LPS challenge. RSV, resveratrol

MCP-1 ($P<0.0001$; Fig. 7F) was increased, while IL-10 levels were decreased ($P<0.0001$; Fig. 7G). In vitro treatment with resveratrol re-established the extracellular content of pro-inflammatory cytokines near to control values, except for IL-10. Resveratrol did not also reduce the mRNA expression of TNF- α and IL-1 β . Regarding cultured astrocytes obtained from control rats, the effects of resveratrol followed a similar profile as described for unstimulated astrocytes in the previous sections.

To demonstrate the potential signaling pathways associated with the resveratrol-mediated glioprotection, we measured the mRNA levels of p21, TLR4, TLR2, p65 NF κ B, SIRT1, Nrf2, and HO-1. Figure 8 displays that astrocyte cultures from LPS-stimulated rats presented an increase in the mRNA expression of p21 (Fig. 8A, $P<0.0001$), TLR2 (Fig. 8C, $P=0.0027$), p65 NF κ B (Fig. 8D, $P=0.0027$), and HO-1 (Fig. 8G, $P<0.0001$) compared to those derived from control animals, while the expression of SIRT1 was decreased (8E, $P=0.0129$). Nrf2 (Fig. 8F) and TLR4 mRNA levels (Fig. 8B) were not changed. The treatment with resveratrol in this experimental model re-established the alterations on NF κ B and SIRT1, and increased Nrf2 and HO-1. However, resveratrol did not change p21 and TLR2 expression. These findings support Nrf2, HO-1, and SIRT1 as classical pathways associated with glioprotective role of resveratrol.

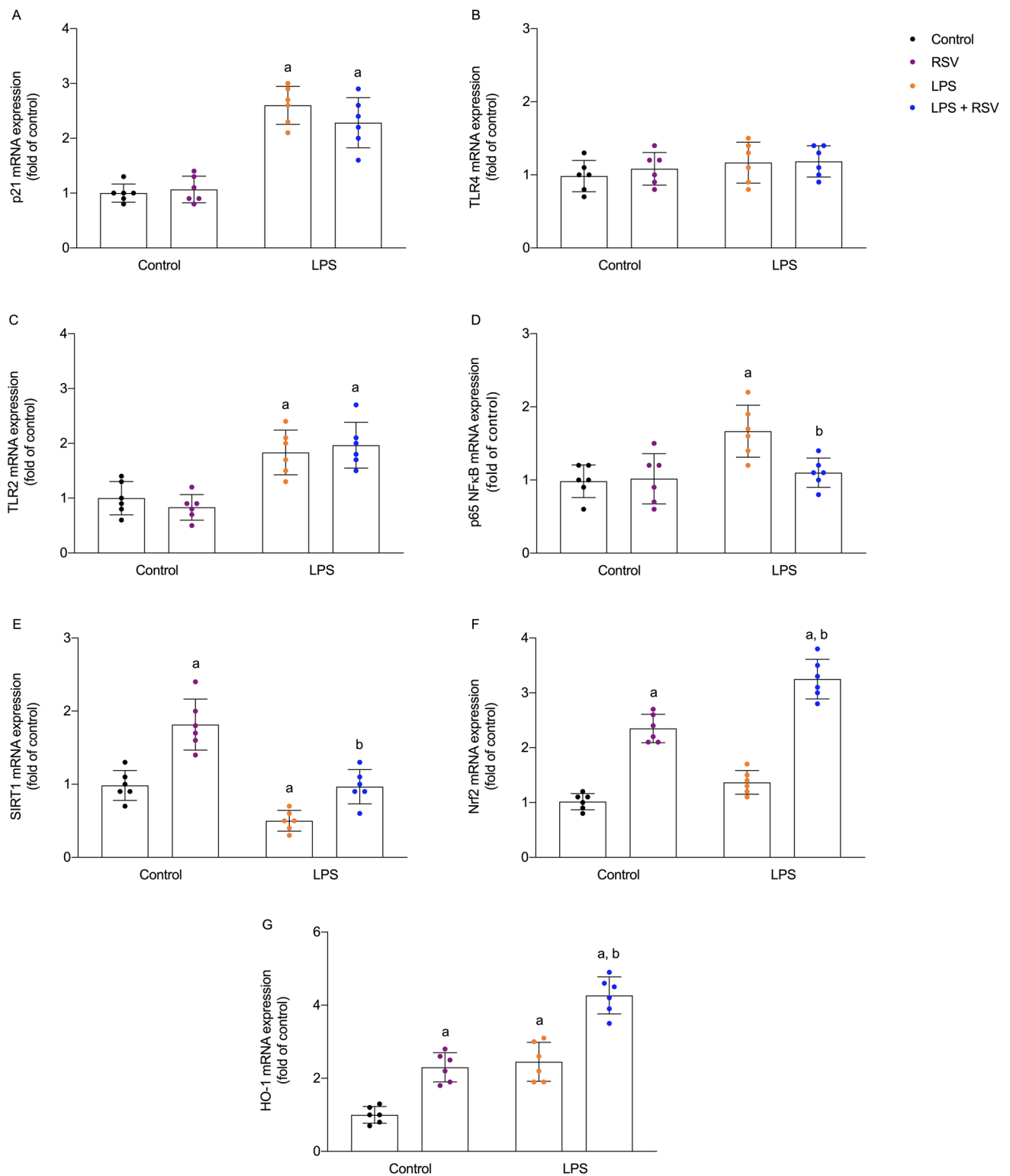
Discussion

Increasing evidence has pointed astrocytes as a significant source of the pro-inflammatory mediators involved in neuro-inflammation [15, 47, 48]. The phenomenon of inflammation is defined as the low-grade inflammation typical of aging, as a result of higher levels of pro-inflammatory markers in cells and tissues of older organisms, which becomes potentially detrimental and acts as a key element for the pathogenesis of neurodegenerative diseases [49, 50]. Importantly, in addition to induce a chronic inflammation, aging can reduce the ability of the cells to adapt to environmental changes and challenging stimuli [17, 20, 48, 51,

52]. Although a previous study from our research group has showed that resveratrol improved some functional parameters and attenuated the release of inflammatory cytokines in mature astrocytes (from 90 and 180 days old Wistar rats) at basal conditions [38], here we studied the potential glioprotective role of resveratrol in chronically inflamed astrocytes from older animals challenged with LPS. For this, primary astrocyte cultures obtained from hippocampus of aged Wistar rats (1-year old) were exposed to LPS (in vitro experimental model), as well as we prepared hippocampal astrocyte cultures from aged rats that received an in vivo LPS injection. Collectively, we demonstrated that resveratrol was able not only to prevent LPS-induced gliotoxicity in vitro, but also to reverse an inflammatory hallmark left in astrocytes by an in vivo LPS injection.

Our previous studies have demonstrated that cultured astrocytes derived from different brain regions (hippocampus, cortex, and hypothalamus) of rats at adult and post-reproductive ages express and/or release increased amounts of pro-inflammatory mediators [17, 18, 39, 51, 54]. Here, we confirm that cultured astrocytes from aged (365 days old) rats in fact presented a marked pro-inflammatory status compared to cultured astrocytes obtained from newborn animals, in addition to have an increased expression of p21 senescence marker, supporting the glial-inflammation process at basal conditions. Moreover, the acute in vitro inflammatory stimulus with LPS produces a powerful inflammatory response in aged astrocytes compared to newborn astrocytes.

Since endogenous protective mechanisms of astrocytes may be insufficient to counteract age-related and exogenously induced inflammation, glioprotective molecules — namely molecules with ability to induce protective responses in glial cells — can represent important strategies to promote a healthier brain aging. In this context, the glioprotective potential of resveratrol in different conditions has received increasingly attention [13, 25, 26, 54, 55]. Here, we observed that resveratrol prevented LPS-induced expression and release of classical cytokines and inflammatory signaling molecules in newborn and aged astrocytes, but especially in aged astrocytes. It is important to note that the upregulation of gene expression can be associated with a further persistent inflammatory response [56, 57]. Reinforcing its glioprotective role, resveratrol was able to prevent the upregulation of p21 in cultured astrocytes stimulated with LPS in vitro, in addition to decrease the expression of p21 by aged astrocytes at basal (unstimulated) conditions. Particularly in the hippocampus, neuroinflammation and cellular senescence can promote several functional alterations that affect neural plasticity, thus contributing to the cognitive decline observed with aging [58, 59]. Thus, these protective actions of resveratrol in aged astrocytes can represent an important strategy to maintain hippocampal functionality.

In vivo LPS experimental model

ROS overproduction and biomolecule damage are processes that can induce cellular dysfunctions and are frequently associated with both aging and inflammatory

responses [2, 60]. Resveratrol also displayed an antioxidant effect in aged astrocytes against LPS *in vitro* exposure by preventing the increase of ROS production and RNA

Fig. 8 Resveratrol changes gene expression of cultured astrocytes obtained from in vivo LPS-injected aged rats. Aged (365 days old) Wistar rats were intraperitoneally injected with LPS or saline (control). After 24 h, astrocyte cultures were prepared and treated with resveratrol (10 μ M; 24 h) at confluence, according to the experimental design depicted in Fig. 6A. The mRNA expressions of p21 (A), TLR4 (B), TLR2 (C), p65 NF κ B (D), SIRT1 (E), Nrf2 (F), and HO-1 (G) were evaluated. Data are presented as mean \pm S.D. and differences among groups were statistically analyzed using two-way analysis of variance (ANOVA), followed by Tukey's test ($n=6$ independent cultures and, at least, duplicate of treatments). Values of $P < 0.05$ were considered significant. *a* refers to statistically significant differences from the control conditions; *b* refers to statistically significant differences from LPS challenge. RSV, resveratrol

oxidation. The close proximity of RNA to mitochondria in the cytosol and its single-stranded structure make RNA vulnerable to oxidation, which can impact protein synthesis and structure, as well as the functions of non-coding RNAs in the brain, and have been linked to neurodegenerative diseases [61]. Moreover, the levels of GSH decrease in astrocyte cultures and in brain tissue with age [17, 51, 62]. Although LPS had no effect, resveratrol could improve GSH levels in aged astrocytes due to the stimulation of GSH synthesis, targeting GCL expression and/or activity, potentially increasing the endogenous ability of these cells to maintain redox homeostasis and respond to oxidative challenges. It is important to note that our group has showed the positive effect of resveratrol on GSH metabolism [25, 35, 38, 63].

Regulation of neurotransmitter systems and trophic signaling are other important functions mediated by astrocytes. Although glutamate is a critical neurotransmitter involved in hippocampal functions such as memory and learning, its excitatory neurotransmission must be tightly regulated to achieve efficient synaptic communication and avoid excitotoxicity [64–66]. In this regard, glutamate transporter downregulation and/or hypofunction in astrocytes impair glutamate uptake and has been naturally observed with aging [17, 51, 64, 67]. In our study, resveratrol improved glutamate uptake, which was correlated with increases in the expression of GLAST and GLT-1 in aged astrocytes in both control and inflammatory conditions. Of note, glutamate uptake can be regulated by several mechanisms, which include gene expression, post-translational modifications, and trafficking to and from the plasma membrane of glutamate transporters [68, 69]. Downregulation of glutamate transporters can be induced by p38 MAPK signaling [70] and may represent a mechanism by which LPS compromises glutamate uptake. Since the activity of glutamate transporters is highly susceptible to oxidation [71], we may also hypothesize that resveratrol improved glutamate uptake due to its antioxidant properties and related pathways, including HO-1 [26]. However, although our study has indicated changes in both mRNA expression and activity of glutamate transporters,

further studies are needed to deeply investigate the mechanisms involved in LPS- and resveratrol-mediated effects on glutamate uptake. The expression and activity of GS, an astrocytic enzyme responsible for conversion of glutamate to glutamine that is highly sensitive to oxidative stress [72], was likewise positively modulated by resveratrol. Trophic support mediated by astrocytes is also essential for hippocampal function and plasticity, but can be compromised by aging and inflammation [13, 17, 73–75]. Similarly to the effects on glutamate metabolism, resveratrol enhanced the release of BDNF and GDNF by aged astrocytes, reinforcing its glioprotective action.

Resveratrol-mediated glioprotection may be associated with several signaling pathways. Nrf2 is a transcription factor that induces the expression of genes associated with antioxidant, anti-inflammatory, and cytoprotective responses, including GCL and HO-1. Nrf-2/HO-1 signaling has been increasingly proposed as an important mechanism underlying the protective roles of resveratrol [25, 26, 63, 76] and may be implicated in the attempt of astrocytes to combat oxidative and inflammatory responses within themselves and neighboring neural cells [77, 78]. Moreover, resveratrol classically activates SIRT1, which can also downregulate inflammatory genes by targeting NF κ B transcriptional activity [79]. Importantly, resveratrol prevented the decrease of the expression of Nrf2, HO-1 and SIRT1 in aged astrocytes exposed to LPS in vitro, in addition to upregulate these genes at basal conditions. Importantly, the effect of resveratrol in promoting not only the direct activation of these proteins, as typically described, but also their gene expression may indicate the induction of an adaptive reprogramming of signaling pathways in astrocytes.

Cultured astrocytes obtained from aged rats can represent a relevant tool for studying cellular, neurochemical, and molecular alterations underlying the aging process in astrocytes, as well as their response to external inflammatory stimuli and potential glioprotective strategies. Importantly, this in vitro model could reproduce major molecular changes observed during an in vivo acute systemic inflammation. In this regard, the expression of pro-inflammatory genes (e.g., cytokines, TLR, p65 NF κ B, and p21) was similarly increased in aged astrocytes exposed to LPS in vitro and in astrocytes obtained from aged rats stimulated with LPS in vivo. Pro-inflammatory markers were also similarly increased in the hippocampal tissue of LPS-injected animals, as well as in their CSF and blood serum. However, it is important to note that the expression of SIRT1 and p21 did not change in hippocampus after LPS challenge. Of note, cellular response to inflammatory stimuli may vary dependent on the presence or absence of other cell types, such as microglial cells [80], as observed with the LPS stimulation in vitro or in vivo and the results from astrocyte cultures and hippocampal tissue. These data are in agreement with a previous study from our

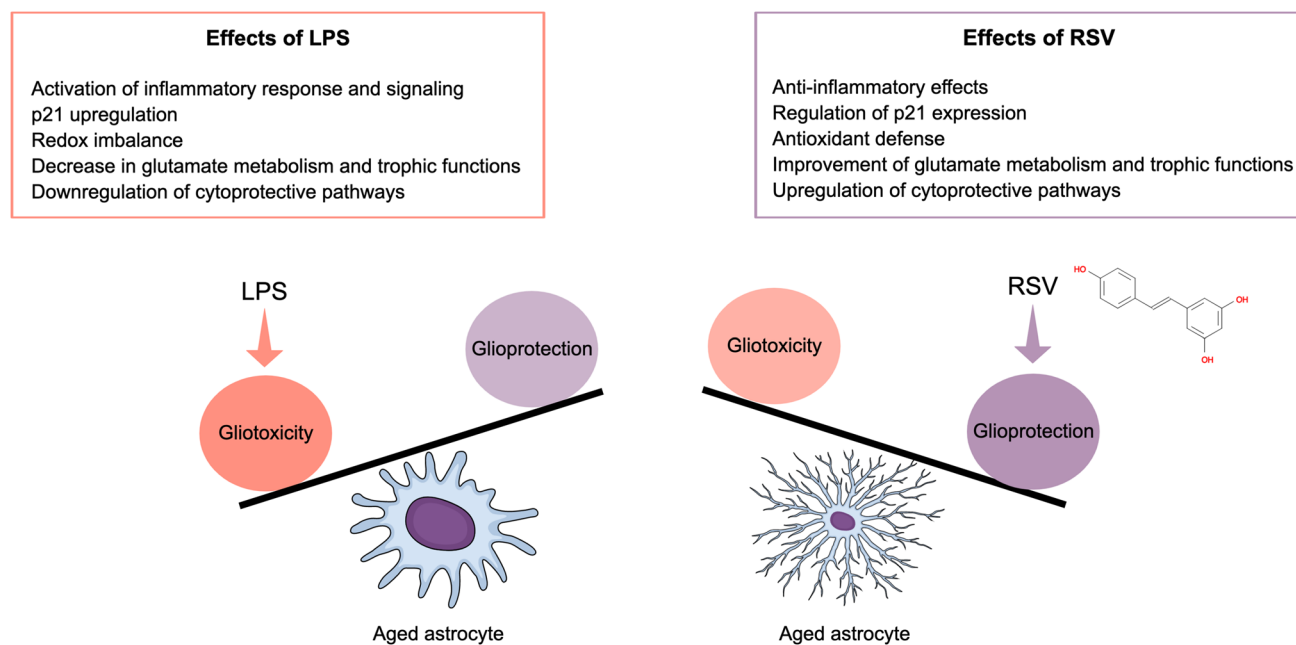


Fig. 9 Schematic illustration of the effects of LPS and resveratrol in primary astrocyte cultures obtained from aged Wistar rats. LPS can induce gliotoxicity (represented by the balance at left with reactive/dysfunctional astrocyte), while resveratrol can promote glioprotection

(represented by the balance at right with ramified/functional astrocyte) by several cellular and molecular mechanisms, which contribute to maintenance of astrocyte functionality. RSV, resveratrol

group that showed an overexpression of inflammatory mediators in cultured astrocytes obtained from animals submitted to a severe systemic inflammation model induced by cecal ligation and perforation [80].

Resveratrol, in turn, also had some different effects depending on the inflammatory experimental model used. In aged astrocytes exposed to LPS *in vitro*, resveratrol was incubated for 1 h prior the addition of LPS and totally prevented its pro-inflammatory effects. However, in cultured astrocytes obtained from aged animals previously subjected to an *in vivo* LPS injection, resveratrol was not able to completely inhibit the pro-inflammatory status presented by astrocytes in culture, particularly regarding to gene expression of TNF- α , IL-1 β , TLR2, and p21, which can indicate a chronic inflammation. These results suggest that resveratrol can act more effectively in preventing rather than reversing inflammatory responses. In addition, anti-inflammatory and cytoprotective molecules (IL-10, Nrf2 and HO-1) were differently modulated in cultured astrocytes exposed to LPS *in vitro* and in astrocytes obtained from animals stimulated with LPS *in vivo*, suggesting different cellular adaptive responses. Interestingly, resveratrol was able to recover the downregulation of SIRT1 in astrocytes obtained from LPS-animals, in addition to potentiate the upregulation of Nrf2 and HO-1, reinforcing its ability to enhance cytoprotective pathways in glial cells. Particularly regarding IL-10, resveratrol increased the release of this cytokine at basal

conditions, but it did not modulate the different effects of LPS (increase of IL-10 in the *in vitro* LPS stimulation and decrease of IL-10 in the *in vivo* LPS stimulation). It is possible to conceive a complex interaction of signaling pathways simultaneously activated in the presence of LPS, resveratrol or both stimuli in the cells, which culminates in a specific astrocyte response. While LPS may stimulate IL-10 expression through NF κ B induction [81], probably as a compensatory mechanism for the acute inflammation, resveratrol may increase IL-10 in a mechanism dependent on HO-1 signaling, as suggested by our data and previous reports [82]. Moreover, it is important to note that the inflammatory response produced by LPS injection *in vivo* can leave inflammatory marks and produce cellular adaptations that could be later observed in astrocytes in culture, in contrast to the *in vitro* stimulation of isolated astrocytes [40]. Astrocytes derived from animals challenged with LPS may also produce and release a different profile of signaling molecules throughout the culture period compared to astrocytes derived from control or naive animals, which we can speculate that impact in the astrocyte gene expression and functionality [83, 84].

Considering that astrocytes have been emerged as potential targets for protection during aging process, with this study we intended to address the effects of resveratrol in the major pathomechanisms associated with aging (Fig. 9). In summary, our data showed that aging can make astrocytes

more responsive to inflammation, contributing to the impairment of astrocyte ability to promote neuroprotection/glioprotection, which may exacerbate neuronal injury and trigger neurodegenerative processes. On the contrary, resveratrol can exert an important glioprotective role, preparing aged astrocytes to better respond to external challenging stimuli and thus contributing to a healthier brain aging.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s12035-021-02664-8>.

Author Contribution AQS and LDB conceptualized the study. LDB, RRSA, FBW, LSM, and LM performed the experiments. LDB and AQS performed statistical analysis and written the original draft of the manuscript. AQS, ATSW, and CAG provided resources and materials/chemicals. All authors revised, edited, and approved the manuscript.

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Data Availability The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code Availability Not applicable.

Declarations

Ethics Approval 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: 21215).

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Conflict of Interest The authors declare no competing interests.

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