Effects of long-term resveratrol treatment in hypothalamic astrocyte cultures from aged rats

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Abstract

Aging is intrinsically related to metabolic changes and characterized by the accumulation of oxidative and inflammatory damage, as well as alterations in gene expression and activity of several signaling pathways, which in turn impact on homeostatic responses of the body. Hypothalamus is a brain region most related to these responses, and increasing evidence has highlighted a critical role of astrocytes in hypothalamic homeostatic functions, particularly during aging process. The purpose of this study was to investigate the in vitro effects of a chronic treatment with resveratrol (1 µM during 15 days, which was replaced once every 3 days), a recognized anti-inflammatory and antioxidant molecule, in primary hypothalamic astrocyte cultures obtained from aged rats (24 months old). We observed that aging process changes metabolic, oxidative, inflammatory, and senescence parameters, as well as glial markers, while long-term resveratrol treatment prevented these effects. In addition, resveratrol upregulated key signaling pathways associated with cellular homeostasis, including adenosine receptors, nuclear factor erythroid-derived 2-like 2 (Nrf2), heme oxygenase 1 (HO-1), sirtuin 1 (SIRT1), proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), and phosphoinositide 3-kinase (PI3K). Our data corroborate the glioprotective effect of resveratrol in aged hypothalamic astrocytes, reinforcing the beneficial role of resveratrol in the aging process.

Keywords Aging · Hypothalamic astrocytes · Resveratrol · Glioprotection

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Introduction

The hypothalamus is an important brain region that controls several physiological processes, including metabolism, reproduction, circadian rhythm, and homeostasis [1]. These intricate and complex processes are controlled by appropriate cellular communication at different levels and cellular circuits, which rely on the proper functioning of astrocytes [2]. In addition, changes in hypothalamic function are closely associated with aging and the progression of neurodegenerative diseases, particularly regarding to energy homeostasis, endocrine and inflammatory responses [3–5]; thus, hypothalamus has been hypothesized to regulate the process of aging of the body.

Astrocytes are abundant cells throughout the central nervous system (CNS) that make intimate contacts with synapses, blood vessels, and other glial cells. Thus, they are involved in many fundamental processes such as synaptic transmission, maintenance of blood–brain barrier (BBB), and sensing nutrients and hormones [6, 7]. Astrocytes also participate in ionic homeostasis and the metabolism

of neurotransmitters, particularly glutamate [8]. In the hypothalamus, all these characteristics are essential for controlling energy homeostasis [9]. However, during metabolic stresses and aging, astrocytes may adopt an activated phenotype, with increased secretion of pro-inflammatory cytokines, excessive production of reactive oxygen/nitrogen species (ROS/RNS), and eventual changes in the expression of glial fibrillary acidic protein (GFAP) [3, 9, 10], which potentially lead to the occurrence of neurotoxicity and neuroinflammation [11].

Resveratrol (3,5,4′-trans-trihydroxy-stilbene) is a polyphenol that naturally occurs in grapes, wines, peanuts, and berries [12, 13] that has been recognized as a multi-target molecule that displays several beneficial effects on the CNS, mainly associated with its antioxidant, anti-inflammatory, and anti-aging properties [14–19]. These effects are associated with different signaling pathways, including nuclear factor erythroid-derived 2-like 2 (Nrf2), heme oxygenase 1 (HO-1), sirtuin 1 (SIRT1), phosphoinositide 3-kinase (PI3K), AMP-activated protein kinase (AMPK), nuclear factor kappa B (NFκB), and adenosine receptors [15, 16, 20, 21], which may directly or indirectly influence cellular metabolism and mitochondrial function. Resveratrol can activate SIRT1 and then upregulate peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) [22, 23]. In mice, chronic treatment with resveratrol had positive effects on metabolic health [13], but to our knowledge, there are no studies about the chronic effects of resveratrol in hypothalamic astrocytes from aged animals.

Regarding to aging process, mature astrocytes present an overall increase in the expression of inflammatory signaling components and a decrease in cytoprotective pathways [3, 24]. Therefore, we hypothesized that these changes in hypothalamic astrocyte could be prevented by long-term exposure of resveratrol, which mediates glioprotection in several experimental models. In this sense, the aim of this present study was to evaluate the effects of chronic resveratrol treatment in the expression of glial markers and molecular pathways associated with inflammatory response and redox homeostasis in hypothalamic astrocyte cultures of aged Wistar rats. With this approach, we reinforced the glioprotective role of resveratrol contributing to characterize its potential mechanisms in the aging process focusing on hypothalamus.

Methods

Reagents

Resveratrol was obtained from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium/F12 (DMEM/F12) and other materials for cell culture, TRIzol reagent, and ELISA kits for tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) were purchased from Gibco/Invitrogen (Carlsbad, CA, USA). High Capacity cDNA Reverse Transcription kit, Taqman Universal PCR Master Mix, and TaqMan® Assays were purchased from Applied Biosystems (Foster City, CA, USA). All other chemicals were purchased from common commercial suppliers.

Animals

Male Wistar rats (24 months old) were obtained from the breeding colony of the Department of Biochemistry (Federal University of Rio Grande do Sul, Porto Alegre, Brazil) and maintained under a controlled environment (12 h light/12 h dark cycle; 22 ± 1 °C; ad libitum access to food and water). All animal experiments were performed by following the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committees of Federal University of Rio Grande do Sul (process number 35387).

Primary hypothalamic astrocyte cultures preparation and maintenance

Hypothalamic astrocyte cultures were performed with our previous publication [3]. Thirty Wistar rats (24 months old) were anesthetized with isoflurane and after the hypothalamus was aseptically dissected, and the meninges removed. The tissue was enzymatically digested in Hank's balanced salt solution (HBSS) containing 0.05% trypsin at 37 °C for 7 min. The tissue was then mechanically dissociated for 7 min using a Pasteur pipette and centrifuged at $100 \times g$ for 5 min. The pellet was resuspended in HBSS and again mechanically dissociated with a Pasteur pipette until complete homogenization, and then centrifuged at $100 \times g$ for 5 min. After mechanical dissociation and centrifugation, the cells were resuspended in DMEM/F12, supplemented with 10% fetal bovine serum (FBS), 15 mM HEPES, 14.3 mM NaHCO$_3$, 1% Fungizone®, and 0.04% gentamicin. Cells were seeded in 6- or 24-well plates pre-coated with poly-L-lysine and cultured at 37 °C in a 5% CO$_2$ incubator. The cells were seeded at a density of approximately $2–4 \times 10^5$ cells/cm^2. 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 3 days. From the second week on, the astrocytes received medium supplemented with 20% FBS until they reached confluence (at approximately the fourth week). Morphological analysis was performed as control of hypothalamic aged astrocyte cultures in agreement with our previous study [3].

Cellular treatments

Long-term treatment of hypothalamic astrocytes with resveratrol (1 μM) was performed for 15 days [25], a half of the time required for cultured astrocytes reach the confluence. From the second week of culture on, resveratrol was added in the culture medium (DMEM/F12 20% FBS), which was replaced once every 3 days until the cells reached confluence, when the analyses were carried out (Fig. 1). Resveratrol was diluted in 0.005% ethanol. It is important to mention that cells exposed to ethanol were not different from those obtained at basal conditions without vehicle, which is in accordance with our previous publication [26].

MTT reduction assay

MTT (methylthiazolyldiphenyl-tetrazolium bromide, Sigma-Aldrich) was added to the culture medium at a concentration of 50 μg/mL and cells were incubated for 3 h at 37 °C in an atmosphere of 5% CO_2. Subsequently, the medium was removed and the MTT crystals were dissolved in dimethyl-sulfoxide. Absorbance values were measured at 560 nm and 650 nm [16].

## Lactate dehydrogenase (LDH) assay

The release of the enzyme LDH was assessed measuring its activity in the culture medium (100 μL) of astrocytes using a commercial UV assay (Bioclin, Brazil).

Extracellular lactate levels

Lactate levels in the extracellular medium were quantified by a commercial kit from Bioclin (Belo Horizonte, MG, Brazil). The results are expressed in mmol/L.

RNA extraction and quantitative RT-PCR

Total RNA was isolated from astrocyte cultures using TRI-zol Reagent. Extracted RNA (1 μg) was submitted to cDNA synthesis by High Capacity cDNA Reverse Transcription Kit. Quantitative PCR determination of the messenger RNAs (mRNAs) encoding GFAP (#Rn00566603_m1), vimentin (#Rn00667825_m1), nestin (#Rn00564394_m1), connexin-43 (Cx43; #Rn01433957_m1), glutamate aspartate transporter (GLAST; #Rn00570130_m1), glutamate transporter 1 (GLT-1; #Rn00691548_m1), aquaporin 4 (AQP4; #Rn00563196_m1), glutamine synthetase (GS; #Rn01483107_m1), p21 (#Rn 00589996_m1), tumor necrosis factor-α (TNF-α; #Rn99999017_m1), interleukin-1β (IL-1β; #Rn00580432_m1), NLR family pyrin domain containing 3 (NLRP3; #Rn04244620_m1), interleukin-10 (IL-10 (#Rn00563409_m1), NFκB p65 (#Rn01502266_ m1), cyclooxygenase 2 (COX2; #Rn01483828_m1), glu-cocorticoid receptor (GR; #Rn00561369_m1), toll-like receptor 4 (TLR4; #Rn00569848_m1), toll-like receptor 2 (TLR2; #Rn02133647_s1), receptor for advanced gly-cation end products (RAGE; #Rn01525753_g1), S100 calcium-binding protein B (S100B; #Rn04219408_m1), high mobility group box 1 (HMGB1; #Rn02377062_g1), adenosine receptor A_1 (#Rn00567668_m1), adenosine receptor A_{2a} (#Rn00583935_m1), adenosine receptor A_{2b} (#Rn00567697_m1), adenosine receptor A_3 (#Rn00563680_ m1), glutamate-cysteine ligase (GCL; #Rn00689046_ m1), superoxide dismutase 1 (SOD1; #Rn00566938_m1), super-oxide dismutase 2 (SOD2; #Rn00690588_g1), PGC-1α (#Rn00580241_m1), Poly (ADP-ribose) polymerase (PARP; #Rn00565018_m1), inducible nitric oxide synthase (iNOS; #Rn00561646_m1), HO-1 (#Rn01536933_ m1), Nrf2 (#Rn00582415_m1), SIRT1 (#Rn01428096_ m1), PI3K (#Rn01769524_m1), AMPK (#Rn00576935_m1), β-actin
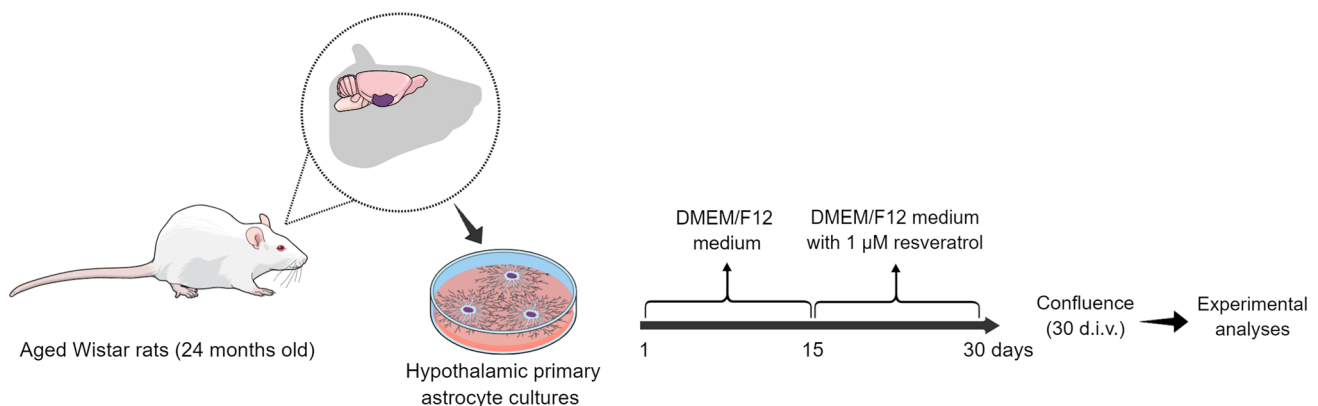
Fig. 1 Timeline of experimental design. The hypothalamus of male Wistar rats (24 months old) was dissected and processed to obtain the primary astrocyte cultures. Astrocytes were maintained in culture medium (DMEM/F12) for 15 days and then resveratrol (1 μM) was resuspended in this medium for the chronic treatment (15 days). *d.i.v.* days in vitro

(#Rn00667869_m1) were performed using the TaqMan real-time RT-PCR system with inventory primers and probes purchased from Applied Biosystems (Thermo Fisher Scientific), as referred for each gene. Target mRNA levels were normalized to β-actin levels. Results were analyzed employing the $2^{-\Delta\Delta Ct}$ method [27].

Inflammatory response measurement

Cytokine levels were measured in the extracellular medium using ELISA kits for TNF-α and IL-1β from Gibco/Invitrogen (Carlsbad, CA, USA). The results are expressed in pg/mL and the average minimum sensitivity of the ELISA kits detection was: 25 pg/mL for TNF-α and 12 pg/mL for IL-1β.

Western blot analysis

Astrocytes were solubilized in a lysis solution containing 4% SDS, 2 mM EDTA, and 50 mM Tris–HCL (pH 6.8). Samples were heated at 100 °C for 10 min, fractionated by SDS-PAGE (30 µg protein per sample) and electro-blotted onto nitrocellulose membranes. Protein loading and electro-blotting efficiency were verified through Ponceau S staining. The membrane was blocked in 5% albumin prepared in Tween-Tris-buffered saline (TTBS; 100 mM Tris–HCl, pH 7.6, containing 70 mM NaCl and 0.1% Tween-20), incubated overnight at 4 °C with the primary antibodies anti-NFκB (1:1000, Cell Signaling, Massachusetts, USA), anti-PGC-1α (1:1000, Invitrogen, Massachusetts, USA), anti-β-actin (1:20,000, ProteinTech Group, Illinois, USA) and washed with TTBS. The membrane was incubated with anti-IgG from rabbit or mouse (1:5000, Cell Signaling, Massachusetts, USA) according to the species that originated the primary antibody linked to peroxidase for 2 h at room temperature and washed with TTBS again. The immunoreactivity was detected by enhanced chemiluminescence using Millipore Immobilon™ Western chemiluminescent HRP substrate in a CCD camera (GE Image Quant LAS4000). Densitometric analysis of the membranes was performed with ImageJ software. Blots were developed to be linear in the range used for densitometry. The representative images of Western blot analysis are in the Supplementary Material (Fig. S1).

Statistical analysis

Data were statistically analyzed using Student's *t*-test. *p* values < 0.05 were considered significant. *Indicates differences between control and resveratrol groups ($n = 10$ independent astrocyte cultures and, at least, duplicate of treatments). All analyses were performed using GraphPad Prism 9.

Results

Resveratrol modulates important glial parameters

First, we tested three concentrations of resveratrol (1, 10 and 100 µM) in accordance with previous studies in astroglial cells [26, 28]. Chronic treatment of aged hypothalamic astrocytes with 1 and 10 µM of resveratrol did not change cellular viability measured by MTT reduction, neither LDH activity (data not shown). However, 100 µM of resveratrol decreased MTT reduction, but not the activity of LDH (data not shown). Therefore, we choose the safely applicable concentration of 1 µM of resveratrol, since it may be more compatible with those obtained in vivo and it is in a range of concentrations that have been used in long-term in vitro studies [25, 29–31]. Moreover, the extracellular levels of lactate, measured as an indicative of metabolic functionality of the cells, were not altered (data not shown).

We then determined the effects of resveratrol on glial markers. Chronic resveratrol exposure decreased the mRNA levels of GFAP ($p < 0.05$; Fig. 2A) but did not change vimentin, nestin, and Cx43 (Fig. 2B–D, respectively), while the expression of AQP4 was decreased ($p < 0.0001$; Fig. 2E). In addition, the main glutamate transporters, GLAST and GLT-1, had a significant increase after resveratrol treatment ($p < 0.05$; Fig. 2F, G). GS, an important enzyme in glutamate metabolism, was significantly increased after resveratrol treatment ($p < 0.0001$; Fig. 2H).

Resveratrol modulates inflammatory response and adenosine receptors

Next, we evaluated the effects of resveratrol on inflammatory response. Resveratrol decreased mRNA levels of p21 ($p < 0.0001$; Fig. 3A), an important regulator of senescence and inflammation. In addition, resveratrol decreased mRNA expression and extracellular levels of TNF-α ($p < 0.05$; Fig. 3B and C, respectively), while decreased only the mRNA expression of IL-1β ($p < 0.0001$; Fig. 3D). The release of IL-1β (Fig. 3E) was not affected. In contrast, resveratrol increased the expression of the anti-inflammatory cytokine IL-10 ($p < 0.0001$; Fig. 3F) and the mRNA expression of NLRP3 (Fig. 3G), which is involved in IL-1β processing, were not affected.

Potentially mediating these inflammatory events, we verified a downregulation of NFκB p65 and COX-2 ($p < 0.05$; Fig. 3H and J, respectively). However, NFκB p65 protein levels were not changed (Fig. 3I). Chronic treatment with resveratrol also modulated the expression

Fig. 2 Changes in classical glial parameters after chronic treatment with resveratrol in cultured hypothalamic astrocytes. The cells were incubated with resveratrol (1 μM) in the culture medium for 15 days and the mRNA expression of GFAP (**A**), vimentin (**B**), nestin (**C**), Cx43 (**D**), AQP4 (**E**), GLAST (**F**), GLT-1 (**G**), and GS (**H**) were evaluated. The data represent the means ± S.D., analyzed by Student's *t*-test. *p* values < 0.05 were considered significant. *Indicates differences between control and resveratrol groups (*n* = 10 independent cultures and, at least, duplicate of treatments). *RSV* resveratrol
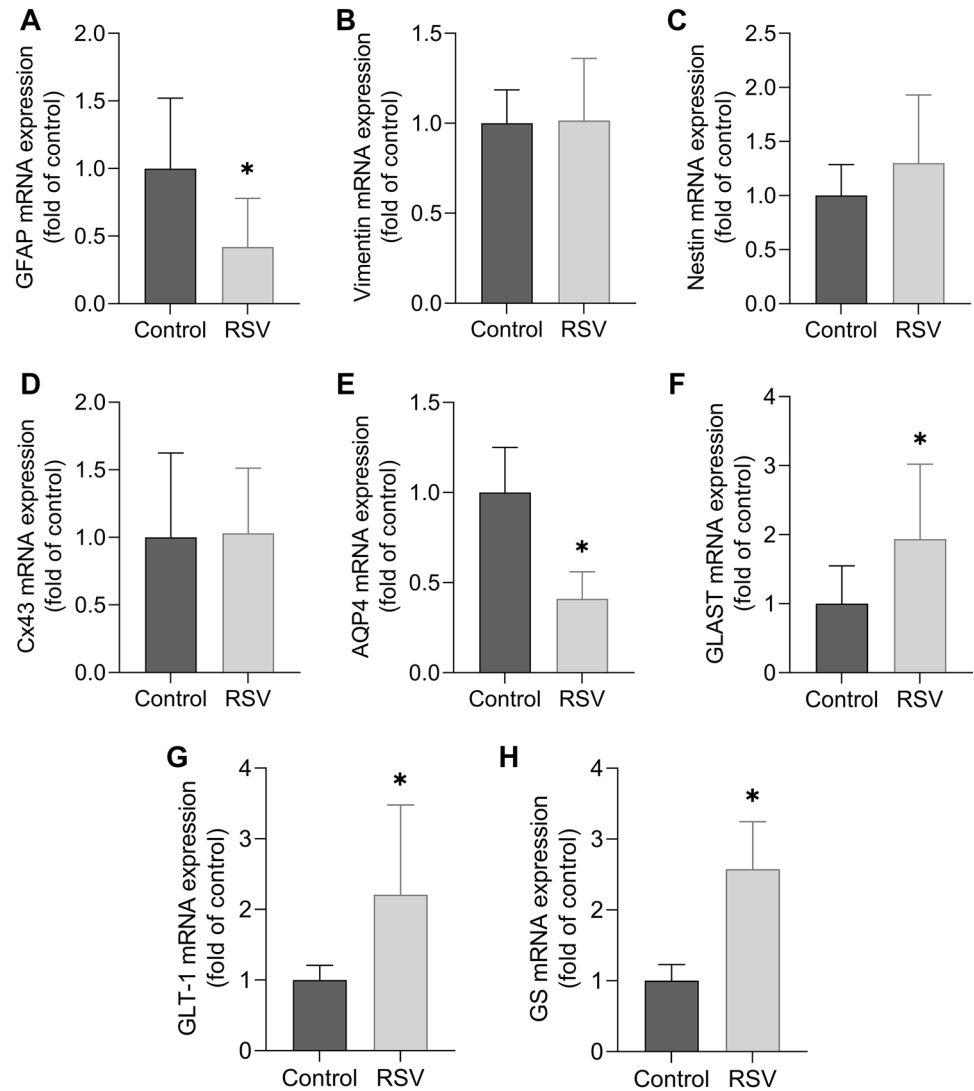
of receptors involved in the regulation of inflammatory responses. GR and TLR4 were downregulated ($p < 0.05$; Fig. 3K and L, respectively), while TLR2 levels were not significantly different (Fig. 3M). Although the mRNA levels of RAGE (Fig. 3N) were upregulated ($p < 0.05$), resveratrol decreased the expression of S100B ($p < 0.05$; Fig. 3O), a potential RAGE ligand, and did not change HMGB1 expression (Fig. 3P).

We also evaluated the expression of adenosine receptors, which are recognized for their important inflammatory and neuromodulatory activities. Resveratrol increased the mRNA levels of A_1, A_{2a}, and A_3 receptors ($p < 0.05$; Fig. 4A–C), but did not change the expression of A_{2b} receptor (Fig. 4D).

Resveratrol changes redox homeostasis

Subsequently, we evaluated the expression of genes associated with redox homeostasis after chronic treatment with resveratrol. We observed an upregulation of GCL, SOD1, SOD2, and PGC-1α ($p < 0.0001$; Fig. 5A–D). The protein levels of PGC-1α were not significantly changed (Fig. 5E). Furthermore, the enzyme PARP and iNOS were downregulated by resveratrol ($p < 0.0001$; Fig. 5F and G, respectively).
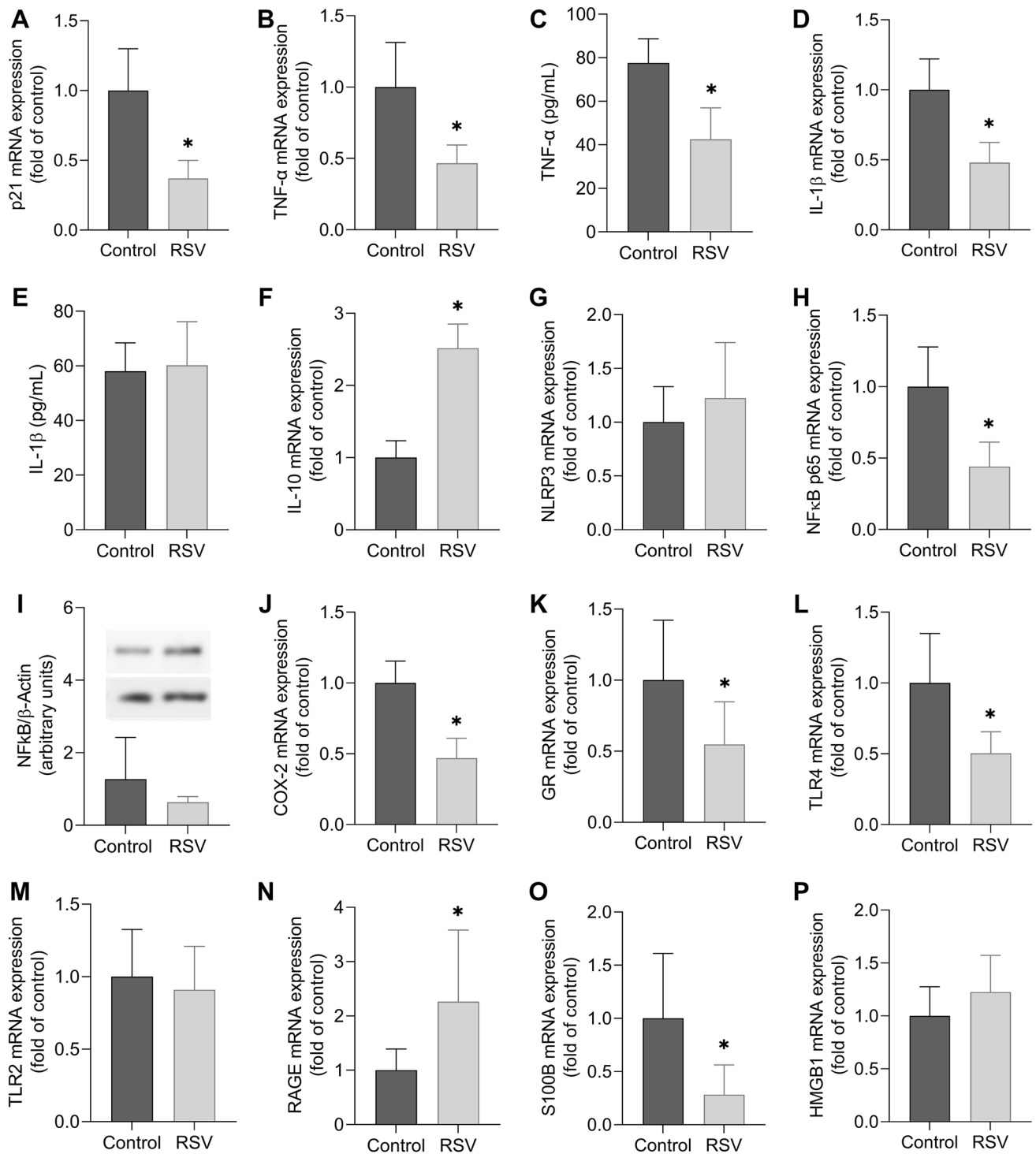
Fig. 3 Effects of chronic resveratrol treatment on inflammatory parameters in cultured hypothalamic astrocytes. The cells were incubated with resveratrol (1 μM) in the culture medium for 15 days and the mRNA of p21 (**A**) and TNF-α (**B**), TNF-α release (**C**), mRNA of IL-1β (**D**), IL-1β release (**E**), mRNA of IL-10 (**F**), NLRP3 (**G**), NFκB (**H**), NFκB protein levels (**I**), mRNA of COX-2 (**J**), GR (**K**), TLR4 (**L**), TLR2 (**M**), RAGE (**N**), S100B (**O**), HMGB1 (**P**) were evaluated. The data represents the means ± S.D., analyzed by Student's t-test. p values < 0.05 were considered significant. *Indicates differences between control and resveratrol groups ($n = 10$ independent cultures and, at least, duplicate of treatments). *RSV* resveratrol
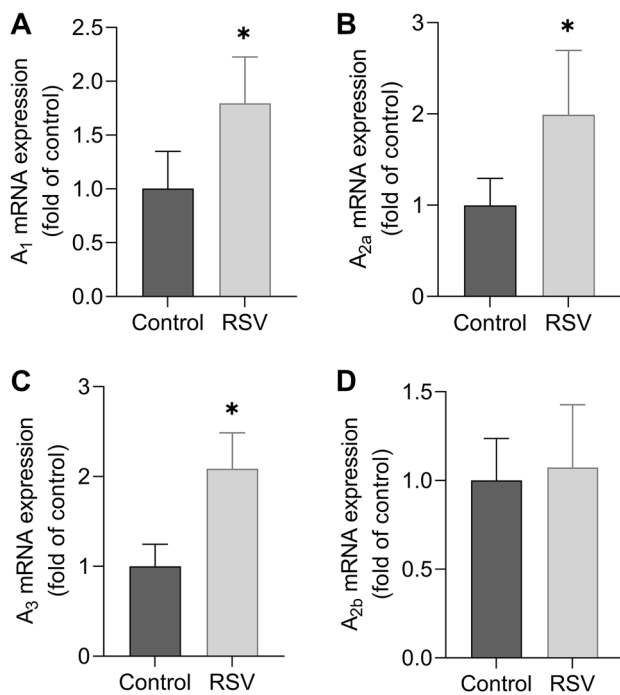
Fig. 4 Effects of chronic resveratrol treatment on adenosine receptors in cultured hypothalamic astrocytes. The cells were incubated with resveratrol (1 μM) in the culture medium for 15 days and the mRNA expressions of A_1 (**A**), A_{2a} (**B**), A_3 (**C**), A_{2b} (**D**) were evaluated. The data represents the means ± S.D., analyzed by Student's t-test. p values < 0.05 were considered significant. *Indicates differences between control and resveratrol groups ($n = 10$ independent cultures and, at least, duplicate of treatments). *RSV* resveratrol

Signaling pathways associated with glioprotective effects of resveratrol

Finally, we analyzed important signaling pathways that can mediate the glioprotective effects of resveratrol. The expression of HO-1 ($p < 0.05$; Fig. 6A), Nrf2 ($p < 0.0001$; Fig. 6B), SIRT1 ($p < 0.05$; Fig. 6C), and PI3K ($p < 0.05$; Fig. 6D) was upregulated by resveratrol. However, resveratrol decreased the mRNA levels of AMPK ($p < 0.05$; Fig. 6E).

Discussion

Aging, a natural biological process, is characterized by a gradual accumulation of oxidized biomolecules and damaged cell organelles, leading to progressive loss of structural and functional integrity of the cells. Metabolism and aging can affect each other mutually; during aging, there are massive changes in body energy metabolism, in which the hypothalamus plays an important role [32]. Accumulating evidence suggests that microinflammatory insults disturb hypothalamic regulation, resulting in metabolic imbalance and aging progression [33]. These processes involve a remodeling of the hypothalamus, which is closely related to astrocytic functionality and changes in gene transcription [3, 19, 34].

Aged human brains display heterogeneous changes in astrocyte morphology and GFAP levels, in addition to
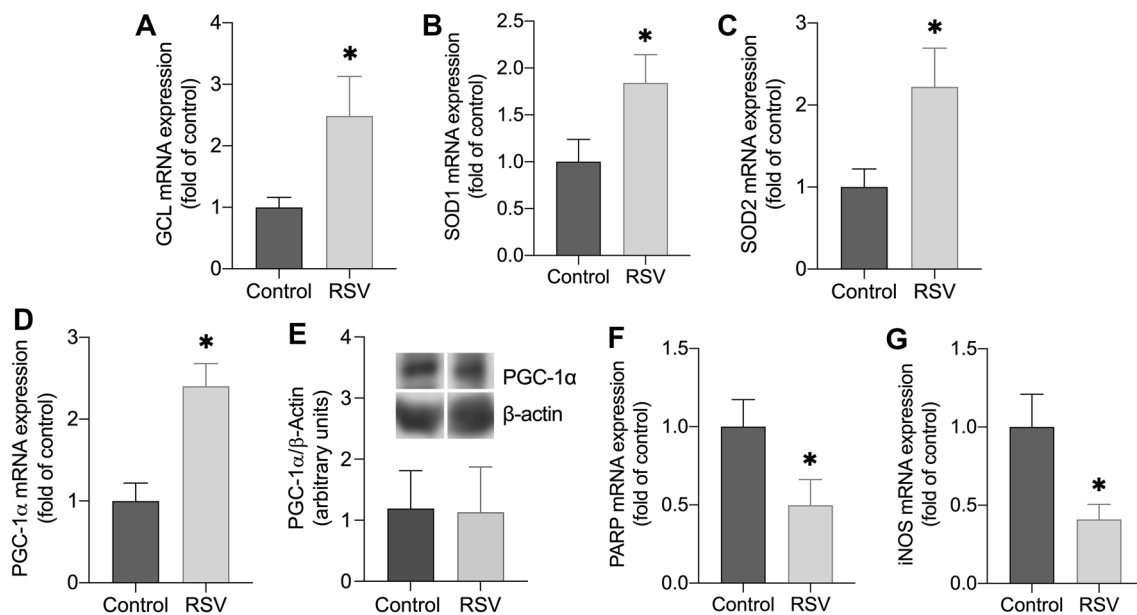
Fig. 5 Changes in redox homeostasis of hypothalamic astrocyte cultures after chronic treatment with resveratrol. The cells were incubated with resveratrol (1 μM) in the culture medium for 15 days. The mRNA expressions of the following enzymes were evaluated: GCL (**A**), SOD1 (**B**), SOD2 (**C**), PGC-1α (**D**), PARP (**F**), and iNOS (**G**), as well as PGC-1α protein levels (**E**). The data represents the means ± S.D., analyzed by Student's t-test. p values < 0.05 were considered significant. *Indicates differences between control and resveratrol groups ($n = 10$ independent cultures and, at least, duplicate of treatments). *RSV* resveratrol

Fig. 6 Glioprotective signaling pathways associated with chronic resveratrol treatment. The cells were incubated with resveratrol (1 μM) in the culture medium for 15 days. mRNA expressions of HO-1 (**A**), Nrf2 (**B**), SIRT1 (**C**), PI3K (**D**), and AMPK (**E**) were evaluated. The data represents the means ± S.D., analyzed by Student's t-test. p values < 0.05 were considered significant. *Indicates differences between control and resveratrol groups ($n = 10$ independent cultures and, at least, duplicate of treatments). *RSV* resveratrol
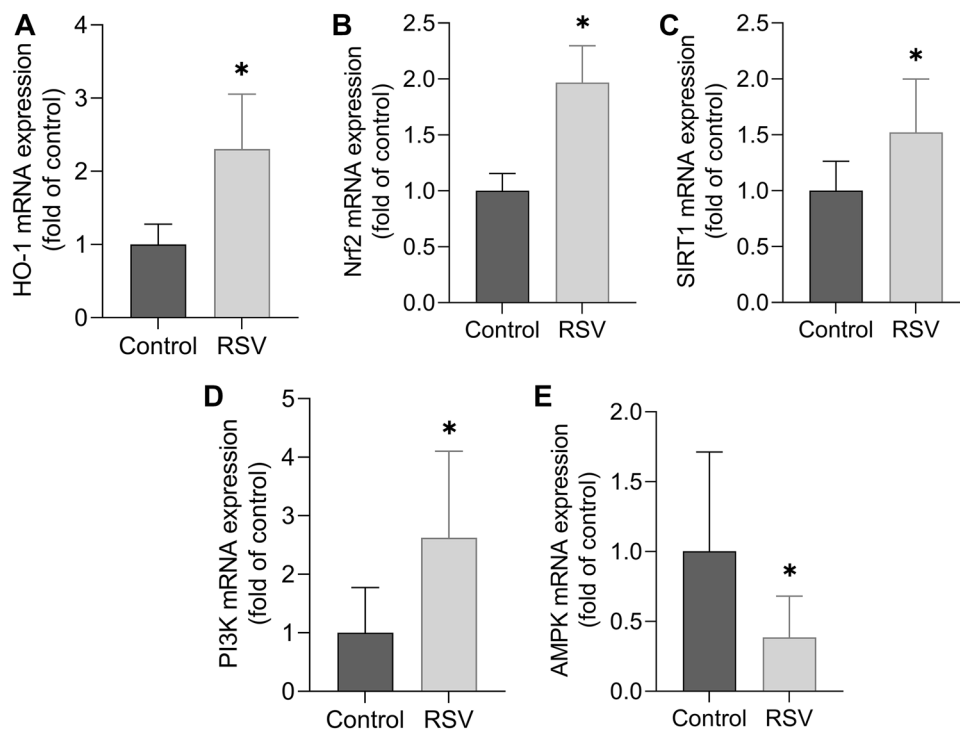
mitochondrial/redox homeostasis dysfunction, neuroinflammation, excitotoxicity, and alterations in glucose metabolism [35, 36]. When we evaluated the glial markers in our study, we found a downregulation of GFAP after chronic resveratrol treatment. Because aging astrocytes have shown increased GFAP expression, which may be associated with reactive/activated astrocytes [37, 38], our finding suggests that chronic resveratrol may exert a protective effect by attenuating aging-related astrocyte activation. Another important functional parameter of astrocytes is AQP4, a water channel protein expressed by these cells [39]. Previously, we showed a higher expression of AQP4 in mature astrocytes compared with neonatal cells [24]. In addition to its role in water homeostasis, AQP4 has been related to neuroinflammation and neurodegenerative processes [40, 41], being the downregulation of AQP4 observed in our study possibly related to the role of resveratrol in maintaining astrocyte homeostasis.

Glutamate is the predominant excitatory neurotransmitter in the CNS. Particularly in the hypothalamus, glutamate has been associated with neuroendocrine regulation and feeding behavior [42]. However, it can be neurotoxic when inappropriately remaining at high levels in the synaptic cleft [43, 44], and hypofunction and downregulation of glutamate transporters may be associated with pathological conditions and aging. GLAST is an important glutamate transporter present in glial cells, but the major glutamate transporter in the brain is GLT-1, responsible for more than 90% of glutamate uptake [44]. Here, we observed that chronic treatment

with resveratrol increased the expression of both GLAST and GLT-1 in aged hypothalamic astrocytes. Resveratrol-induced improvement in glutamate uptake has been reported in previous studies by our group [14, 28, 45], which may be related to the increased expression of glutamate transporters found in the current study. It is important to note that the activity of glutamate transporters can be impaired by oxidative damage [46] and inflammatory responses, which are also attenuated by resveratrol [47].

In addition to increase glutamate transporters, resveratrol also upregulated the expression of GS, a specific astrocytic enzyme responsible for the conversion of glutamate in glutamine. Glutamine is then forwarded to neurons as an intermediate for recycling glutamate [48]. Our previous works have demonstrated that GS expression decreased in cultured astrocytes in an age-dependent manner, demonstrating that resveratrol may act as a preventive factor in this process [3, 49]. Interestingly, glutamate metabolism can be modulated by glucocorticoids; they are related to decreased glutamate uptake [50] and GS activity and expression [51] by astrocytes. We observed a downregulation of glucocorticoid receptors (GR) after resveratrol treatment, which may represent an additional mechanism by which resveratrol exerts the homeostatic role in glutamate metabolism. In addition, glucocorticoid actions in the hypothalamus may provide an integrative signal linking stress with the regulation of energy homeostasis [52].

With the progression of aging, the expression of senescence markers, including p21, also increases. This protein

promotes cell cycle arrest in senescent cells, and the inhibition of this marker is recognized as an important way to treat age-related pathologies [53]. Importantly, we observed a downregulation of p21 by the chronic treatment with resveratrol in hypothalamic astrocytes. Aging progression is also characterized by inflammatory responses throughout the body, according to the concept of "inflammaging" [54]. With this regard, hypothalamic astrocytes from adult and aging animals have presented a pro-inflammatory profile [3], which may lead to a chronic inflammatory background in the hypothalamus [33]. The NFκB pathway is an important player in astrocyte reactivity [32] and age-related hypothalamic inflammation [55], while inhibition of NFκB has been associated with improvement of various aspects of metabolic conditions. Because many inflammatory cytokines are induced through this transcription factor, the downregulation of NFκB p65 expression by resveratrol may be effective in suppressing hypothalamic microinflammation [33]. In agreement with that, astrocytes chronically treated with resveratrol presented a downregulation of several pro-inflammatory mediators, including TNF-α, IL-1β, and COX-2, and an upregulation of the anti-inflammatory cytokine IL-10. Of note, adenosine receptors play neuromodulatory and neuroinflammatory functions [56, 57] that have been associated with resveratrol-mediated anti-inflammatory activity in a previous study by our group [16]. Here, adenosine receptors were also positively modulated by resveratrol chronic treatment, reinforcing their role in the glioprotective effects of resveratrol.

In addition to produce and release a wide range of inflammatory mediators, astrocytes can also respond to them during immune responses through the expression of pattern recognition receptors [58]. In neurodegenerative diseases and CNS injuries, endogenous and exogenous ligands trigger TLR signaling, leading to the transcription of inflammatory cytokines [59]. Thus, the downregulation of TLR4 after treatment with resveratrol may be associated with a negative modulation of inflammatory markers. Moreover, resveratrol decreased S100B, which can act as an extracellular factor that engages RAGE [60]. Activation of RAGE by its ligands induces the release of cytokines, interleukins, and increased reactive oxygen species production in glial cells [61]. However, recent studies demonstrate that RAGE activation also contribute to neuronal-astrocytic communication [62] and promote changes in redox regulation.

Although reactive oxygen/nitrogen species (ROS/RNS) carry out modulatory functions in physiological conditions, excessive production of these species can contribute to the development and progression of pathological processes [63]. Aging process leads to mitochondrial dysfunction and imbalance of redox homeostasis, with significant involvement of oxidative and nitrosative stresses [36, 64]. With this regard, glial cell-related antioxidant defenses display

important roles for the protection and repair of the brain, and are essential for controlling energy homeostasis [9]. PGC-1α is a transcription co-activator for nuclear receptors and plays a fundamental role in the control of cellular energy metabolism, redox homeostasis, and neuroinflammation [23, 65]. The astrocytic antioxidant system also involves the synthesis of GSH, which depends on the enzyme GCL. Importantly, neurons are dependent on astrocytic GSH to their own antioxidant defense [66]. In our study, we verified an increase in the expression of PGC-1α and GCL, as well as SOD1 and SOD2, indicating an improvement of antioxidant defenses in hypothalamic astrocytes from aged rats by the chronic treatment with resveratrol. In addition, aging brains show higher levels of PARP and iNOS [64, 67], which was downregulated in our experimental model, reinforcing the antioxidant action of the chronic treatment with resveratrol.

Oxidative stress and inflammation are closely related processes, which are connected by important signaling pathways, such as NFκB and Nrf2 [68]. Signaling pathways act as key regulators of cell survival and responses to different physiological and pathological. Resveratrol is effective to prevent age-related functional alterations of astrocytes by different signaling pathways, including Nrf2/HO-1, PI3K/Akt, AMPK, and NFκB [15, 16, 20, 21]. Nrf2 is an essential transcription factor responsive to metabolic, inflammatory, and redox signals, acting as a regulatory mechanism for the homeostasis of these systems. HO-1, one of the genes regulated by Nrf2, acts to produce cellular responses against stressful conditions [20, 69]. Our data corroborate with these mechanisms since chronic treatment with resveratrol was able to increase the expression of Nrf2 and HO-1. SIRT1 and PI3K, which represent important metabolic effectors, were also upregulated by resveratrol, thus potentially participating in the protective response induced in hypothalamic astrocytes [70, 71]. However, some limitations of this study should be mentioned, including the differences between gene expression and protein levels of NFκB and PGC-1α, probably due to their different regulatory mechanisms.

In addition, data from cultured astrocytes derived from Wistar rats chronically treated with resveratrol will confirm and expand the knowledge about the role of resveratrol on hypothalamus. In line with this, changes in hypothalamus strongly interfere with brain homeostasis, being a critical point in development and progression of age-dependent brain dysfunctions. Therefore, hypothalamic astrocytes may emerge as a therapeutic target for understanding the brain aging under physiological and pathological conditions.

Finally, resveratrol is a recognized anti-inflammatory and antioxidant polyphenol with beneficial effects described in many tissues, but the focus of several studies with this compound is based on the strategy of acute treatments. If we consider long-term treatments, they can have further beneficial effects as a preventive approach.
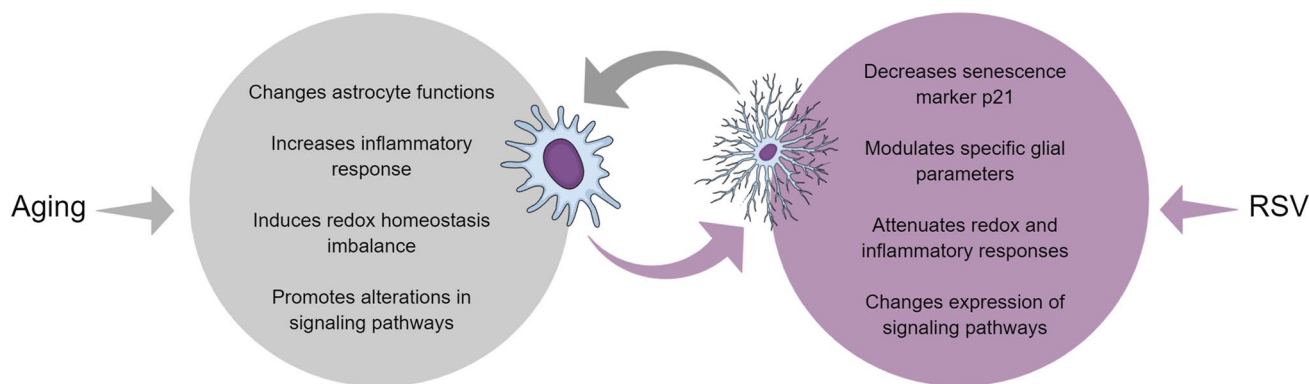
Fig. 7 Glioprotective effects of long-term resveratrol treatment. Schematic illustration summarizing the effects of aging and chronic treatment with resveratrol (1 μM) for 15 days in hypothalamic astrocytes of aged Wistar rats (24 months old). Chronic treatment attenuated the gliotoxicity caused by the aging process in astrocytes, playing a homeostatic role in the hypothalamus. *RSV* resveratrol

Clinical evidence of food or nutrient supplements chronically administered in patients suggest that diets rich in polyphenols, for example the Mediterranean diet, could be an effective nutritional strategy [72]. Therefore, in the near future, our findings might contribute for understanding the effects of resveratrol on hypothalamus, including for clinical studies focusing on metabolism and aging. Considering the effects associated with aging on hypothalamic function (Fig. 7), such as oxidative stress and inflammation, it is reasonable to assume that long-term resveratrol treatment could help with these age-dependent changes in astrocytes and consequently the age-related diseases.

Declarations

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