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Adenosine receptors as a new target for resveratrol-mediated glioprotection

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ABSTRACT

Resveratrol, a natural polyphenolic compound, has been studied as a neuroprotective molecule. Our group has demonstrated that such effect is closely associated with modulation of glial functionality, but the underlying mechanisms are not fully understood. Because astrocytes actively participate in the brain inflammatory response, and activation of adenosine receptors can attenuate inflammatory processes, the aim of this study was to investigate the role of adenosine receptors as a mechanism for resveratrol glioprotection, particularly regarding to neuroinflammation. Therefore, primary astrocyte cultures were co-incubated with resveratrol and selective antagonists of A_1, A_{2A}, and A_3 adenosine receptors, as well as with caffeine (a non-selective adenosine receptor antagonist), and then challenged with bacterial inflammogen lipopolysaccharide (LPS). Caffeine and selective adenosine receptor antagonists abolished the anti-inflammatory effect of resveratrol. In accordance with these effects, resveratrol prevented LPS-induced decrease in mRNA levels of adenosine receptors. Resveratrol could also prevent the activation of pro-inflammatory signaling pathways, such as nuclear factor κB (NFκB) and p38 mitogen-activated protein kinase (p38 MAPK) in a mechanism dependent on adenosine receptors. Conversely, trophic factors and protective signaling pathways, including sirtuin 1 (SIRT1), nuclear factor erythroid 2-related factor 2 (Nrf2), heme oxygenase-1 (HO-1), and phosphoinositide 3-kinase (PI3K)/Akt were positively modulated by resveratrol in both LPS-stimulated and unstimulated astrocytes, but adenosine receptor antagonism did not abrogate all effects of resveratrol. To our knowledge, our data provide the first evidence that adenosine receptors are involved in the anti-inflammatory activity of resveratrol in astrocytes, thus exerting an important role for resveratrol-mediated glioprotection.

1. Introduction

Astrocytes are glial cells that actively participate in synaptic information transmission and plasticity, regulating neurotransmitter metabolism, as well as provide metabolic, trophic and antioxidant support to neurons, maintaining brain homeostasis [1–3]. Additionally, astrocytes are immunocompetent players, releasing a wide array of inflammatory mediators under pathological or injury conditions, including pro- and anti-inflammatory cytokines and chemokines [4–6]. Because astrocytes express toll-like receptors (TLR), including TLR2 and TLR4, they are able to respond to bacterial lipopolysaccharide (LPS), the main component of outer membrane of Gram-negative bacteria, which has been widely used to experimentally study inflammatory response, including in the central nervous system (CNS) [7–9]. LPS stimulates the synthesis and release of pro-inflammatory cytokines through different signaling pathways, including mitogen-activated protein kinases (MAPK) and nuclear factor κB (NFκB). In addition, LPS can induce nitrosative/oxidative stress as a result of exacerbated production of nitric oxide (NO) and reactive oxygen species (ROS) [10–12]. In line with this, increasing evidence has showed that astrocyte

Abbreviations: A_1, adenosine receptor A_1 subtype; A_{2A}, adenosine receptor A_{2A} subtype; A_{2B}, adenosine receptor A_{2B} subtype; A_3, adenosine receptor A_3 subtype; AMPK, adenosine monophosphate-activated protein kinase; BDNF, brain-derived neurotrophic factor; COX-2, cyclooxygenase-2; CNS, central nervous system; ERK, extracellular signal-regulated kinase; GDNF, glial-derived neurotrophic factor; HO-1, heme oxygenase-1; iNOS, inducible nitric oxide synthase; IL-1β, interleukin-1β; IL-6, interleukin-6; IL-10, interleukin-10; IL-18, interleukin-18; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein-1; mTOR, mammalian target of rapamycin; NFκB, nuclear factor κB; NO, nitric oxide; NOX, NADPH oxidase; Nrf2, nuclear factor erythroid 2-related factor 2; PI3K, phosphoinositide 3-kinase; p38 MAPK, p38 mitogen-activated protein kinase; ROS, reactive oxygen species; SIRT1, sirtuin 1; SOCS, suppressor of cytokine signaling; TLR, toll-like receptors; TNF-α, tumor necrosis factor-α

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overactivation is closely related with neuroinflammation and, consequently, neurodegeneration [13].

Resveratrol (3,5,4′-trans-trihydroxy-stilbene) is a polyphenol that naturally occurs in grapes, wines, peanuts and berries. This compound is well recognized for its antioxidant, anti-inflammatory and neuroprotective effects [14,15]. In this regard, many beneficial actions of resveratrol in the CNS can be attributed to its ability to modulate and protect glial cells, consequently preserving functional integrity of the brain [16–19]. The anti-inflammatory activity of resveratrol in glial cells has been described and may be associated with different signaling pathways, including nuclear factor erythroid 2-related factor 2 (Nrf2), heme oxygenase-1 (HO-1), p38 and extracellular signal-regulated kinase (ERK) MAPK [7,16,20,21]. Although the underlying mechanisms are not fully understood, previous studies have shown that resveratrol can modulate the adenosinergic system in a variety of tissues, including cardiovascular and brain [22–25].

Adenosine receptors (A_1, A_{2A}, A_{2B} and A_3) belong to the G protein-coupled receptor family, which are classically activated by adenosine, a purine nucleoside that is the final product of ATP degradation [26]. Adenosinergic signaling is involved in physiological and pathological processes in several tissues, including the CNS, where it plays an important role in neuromodulation and can control neuronal dysfunction and degeneration [27–30]. Moreover, adenosinergic system is able to modulate glial responses, including those related to inflammation [31–35]. Caffeine is a well-known antagonist of adenosine receptors, in particular A_1 and A_{2A} subtypes, exerting its biological effects due to removal of the adenosinergic signaling [36].

Considering the inflammatory response induced by LPS, the crucial role of astrocytes responding to inflammatory stimuli, and the protective effects of resveratrol, particularly its anti-inflammatory activity, the aim of this study was to investigate the role of adenosine receptors, using caffeine and other selective antagonists, as a putative mechanism of resveratrol glioprotection. We reported that LPS challenge decreased the expression of adenosine receptors in cultured astrocytes, while resveratrol induced an upregulation, preventing the effect of LPS. Adenosine receptor antagonists abolished the anti-inflammatory effect of resveratrol against LPS, including the attenuation of the transcriptional activity of NFκB and p38 MAPK immunocontent. Additionally, adenosine receptor antagonists blocked the positive effect of resveratrol on neurotrophic factor release and on important protective signaling pathways, such as sirtuin 1 (SIRT1), Nrf2, HO-1, and phosphoinositide 3-kinase (PI3K)/Akt. On the other hand, caffeine did not affect the antioxidant role of resveratrol. Our data provide the first evidence that adenosine receptors might be crucial to the molecular mechanisms underlying the anti-inflammatory and glioprotective effects of resveratrol.

2. Materials and methods

2.1. Reagents

Resveratrol, LPS, caffeine, 5-diphenyltetrazolium bromide (MTT), NADP/NADPH, lucigenin, propidium iodide (PI), superoxide anion assay, phospho-p38 MAPK and phospho-Akt Ser^{473} kits were obtained from Sigma-Aldrich (St. Louis, MO, USA). 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX), 4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM 241385), *N*-[9-Chloro-2-(2-furanyl)[1,2,4]-triazolo[1,5-*c*]quinazolin-5-yl]benzene acetamide (MRS 1220) were purchased from Tocris (Bristol, UK). ELISA kits for IL-1β, IL-18, IL-10, MCP-1, BNDF and NFκB p65, Dulbecco's Modified Eagle's Medium (DMEM) and other materials for cell cultures were purchased from Gibco/Invitrogen (Carlsbad, CA, USA). GDNF ELISA kit was obtained from R&D Systems (Minneapolis, MN, USA) and ELISA kit for TNF-α from PeproTech (Rocky Hill, NJ, USA). Phospho-PI3K p85 $Tyr^{467/199}$ ELISA kit was obtained from Abcam (Cambridge, MA). Anti-HO-1 and anti-actin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Merck Millipore (Darmstadt, Germany), respectively. All other chemicals were purchased from common commercial suppliers.

2.2. Animals

Newborn Wistar rats were obtained from our breeding colony (Department of Biochemistry, UFRGS, Porto Alegre, Brazil), 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).

2.3. Primary cortical astrocyte cultures preparation and maintenance

Cortices of newborn Wistar rats (1–2 days old) were removed and mechanically dissociated in Hanks' balanced salt solution (HBSS) and left for decantation for 20 min. Supernatant was collected and centrifuged for 5 min (400*g*). After, the pellet was resuspended in DMEM [10% fetal bovine serum (FBS), 15 mM HEPES, 14.3 mM $NaHCO_3$, 1% fungizone and 0.04% gentamicin] and cells were cultured at 37 °C in atmosphere with 5% of CO_2. The first medium exchange occurred 24 h after obtaining the culture. The medium change occurred once every four days until the cells reached the confluence (approximately 14 days *in vitro*) [37].

2.4. Cellular treatments

After the cells reached confluence, the culture medium was exchanged with serum-free DMEM and the dose response of LPS on cell viability and integrity was evaluated after incubating the cells with different concentrations of LPS (1 and 10 μg/mL) for 3 and 24 h. Following, in accordance with previous reports from our group [16,17,38], cells were pre-incubated in the absence or presence of 100 μM resveratrol for 1 h. After this pre-incubation, 1 μg/mL LPS (previously determined from experiments of cellular viability/integrity) was added for 3 or 24 h (resveratrol was maintained). Additionally, to verify the role of caffeine (an adenosine receptor antagonist) on glial functions, astrocytes were co-incubated with resveratrol (100 μM) and caffeine in accordance with a previous study [10], before the LPS treatments, using the same conditions described above. To ensure the involvement of adenosine receptors, astrocytes were also incubated with selective antagonists in the presence of LPS and resveratrol: DPCPX (100 nM), an A_1 antagonist; ZM 241385 (50 nM), an A_{2A} antagonist; MRS 1220 (1 μM), an A_3 receptor antagonist. During all procedures, the cells were maintained at 37 °C in an atmosphere with 5% of CO_2.

2.5. MTT reduction assay

MTT was added to the medium at a concentration of 50 μg/mL and cells were incubated for 30 min 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 [20]. The results are expressed as percentages relative to the control conditions.

2.6. Propidium iodide (PI) incorporation

Flow cytometry analysis was used to assess the integrity of plasma membrane by determining the ability of cells to exclude PI [39]. Briefly, after the treatment with LPS for 3 or 24 h, cells were trypsinized and incubated with 1 μg/mL PI solution at room temperature in the dark for

30 min. Following, the cells were washed twice with PBS and centrifuged at 1000*g* for 10 min at 4 °C to remove the free PI. The level of PI incorporation, indicative of cell death, was determined using flow cytometry (Guava® easyCyte, Merck Millipore, Burlington, MA, EUA). PI dye was excited at 488 nm.

2.7. Inflammatory response measurement

Cytokine levels were measured in the extracellular medium using ELISA kits for tumor necrosis factor-α (TNF-α), from Peprotech (Rocky Hill, NJ, USA), interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-18 (IL-18), interleukin-10 (IL-10) and monocyte chemoattractant protein-1 (MCP-1/CCL2), from Thermo Scientific (Carlsbad, CA, USA). The results are expressed in pg/mL and the average minimum sensitivity of the ELISA kits detection was: 25.0 pg/mL for TNF-α; 12 pg/mL for IL-1β; 16 pg/mL for IL-6; 4 pg/mL for IL-18; 3 pg/mL for IL-10 and 5 pg/mL for MCP-1.

2.8. RNA extraction and quantitative RT-PCR

Total RNA was isolated from astrocyte cultures using TRIzol Reagent (Invitrogen, Carlsbad, CA). The concentration and purity of the RNA were determined spectrophotometrically at a ratio of 260:280. Then, 1 μg of total RNA was reverse transcribed using Applied Biosystems™ HighCapacity complementary DNA (cDNA) Reverse Transcription Kit (Applied Biosystems, Foster City, CA) in a 20 μL reaction according to manufacturer's instructions. The messenger RNA (mRNA) encoding TNF-α (#Rn99999017_m1), IL-1β (#Rn00580432_m1), TLR2 (#Rn02133647_s1), TLR4 (#Rn00569848_m1), NFκB (#Rn01399572_m1), adenosine receptors A_1 (#Rn00567668_m1), A_{2A} (#Rn00583935_m1), A_{2B} (#Rn00567697_m1) and A_3 (#Rn00563680_m1), SIRT1 (#Rn01428096_m1), Nrf2 (#Rn00582415_m1), HO-1 (#Rn01536933_m1), PI3K (#Rn01769524_m1), Akt (#Rn00442194_m1) and β-actin (#Rn00667869_m1) were quantified using the TaqMan real-time RT-PCR system using inventory primers and probes purchased from Applied Biosystems. The mRNA quantification for the genes encoding cyclooxygenase-2 (COX-2: forward 5′-GATTGACAGCCCACCAACTT-3′ and reverse 5′-CGGGATGAACTCTC TCCTCA-3′), inducible nitric oxide synthase (iNOS: forward 5′-GGCA GCCTGTGAGACCTTTG-3′ and reverse 5′-GAAGCGTTTCGGGATCT GAA-3′) and β-actin (forward 5′-CAACGAGCGGTTCCGAT-3′ and reverse 5′-GCCACAGGATTCCATACCCA-3′) was performed using primers pairs as sequence described and Power SYBR Green PCR Master Mix (Invitrogen). Quantitative RT-PCR was performed using the Applied Biosystems 7500 Fast system. No-template and no-reverse transcriptase controls were included in each assay, producing no detectable signal during the 35–40 cycles of amplification. Target mRNA levels were normalized to β-actin levels and expressed relative to the levels in control astrocytes using the $2^{-\Delta\Delta Ct}$ method [40].

2.9. Western blotting analysis

Astrocytes were lysed in a solution containing 4% SDS, 2 mM EDTA and 50 mM Tris–HCl (pH 6.8). Samples were separated by SDS/PAGE (20 μg protein per sample), and transferred to nitrocellulose membranes, which were blocked with 4% albumin and then incubated overnight (4 °C) with anti-HO-1 (1:1000) or anti-actin (1:5000). Actin was used as a loading control. Then, the membranes were incubated with a peroxidase-conjugated anti-immunoglobulin (IgG) at a dilution of 1:10,000 for 2 h. Chemiluminescence signal was detected in an Image Quant LAS4010 system (GE Healthcare) using an ECL kit [41]. The results are expressed as percentages relative to control conditions.

2.10. NFκB levels

The levels of NFκB p65 in the nuclear fraction, which had been isolated from lysed cells with 1% Igepal CA-630 and centrifugation (following manufacturer's instructions), and measured using an ELISA commercial kit from Invitrogen (Carlsbad, CA, USA). The ELISA kit detects a minimum of 50.0 pg/mL. The results are expressed in pg/mL.

2.11. p38 MAPK levels

The phospho-p38 MAPK levels were detected using an ELISA commercial kit from Sigma-Aldrich (St. Louis, MO, USA). These levels were evaluated in the cell lysate suspended in a specific buffer from the ELISA kit. The ELISA kit detects a minimum of 2.5 pg/mL and the results are expressed in pg/mL.

2.12. Phospho-PI3K and Akt levels

Phosphorylated forms of PI3K (phospho-PI3K p85 $Tyr^{467/199}$) and Akt (phospho-Akt Ser^{473}) were evaluated using ELISA kits from Abcam (Cambridge, MA) and Sigma-Aldrich (St. Louis, MO, USA), respectively, following manufacturers' instructions. The results are expressed as percentages relative to control conditions.

2.13. NADPH oxidase (NOX) activity

NOX activity was measured in cell lysate suspended in a sodium phosphate buffer with 140 mM KCl using a modified assay [42]. Briefly, this luminescence assay used lucigenin as the electron acceptor generated by the NADPH oxidase complex. NADPH oxidase assay solution with 5 μM of lucigenin was used and the concentration of NADPH (1 μM–1 mM), used as the substrate, fell well within the linear range of the assay. The data were converted to relative light units/min/mg of protein, using a standard curve generated with xanthine/xanthine oxidase. Lucigenin activity (light units/min/mg of protein) of control cells was arbitrarily set at 100%. The results are expressed as percentages relative to the control conditions.

2.14. Cellular superoxide levels

Cellular superoxide levels were determined using the superoxide anion assay kit from Sigma (St. Louis, MO, USA). The kit method is based on the oxidation of luminol by superoxide anions resulting in the formation of chemiluminescence light. The chemiluminescence measurement in lysed cells increases with superoxide formation. The control cells were arbitrarily set at 100%. The kit includes a superoxide anion producing system (xanthine/xanthine oxidase) for a positive control and the superoxide dismutase enzyme for the repression of the system, used as a negative control. The results are expressed as percentages relative to the control levels.

2.15. Trophic factors measurement

Brain-derived neurotrophic factor (BDNF) and glial-derived neurotrophic factor (GDNF) levels were measured in the extracellular medium, using commercial ELISA kits from Invitrogen (Carlsbad, CA, USA) for BDNF and from R&D Systems (Minneapolis, MN, USA) for GDNF. The results are expressed in pg/mL. The ELISA kits detect a minimum of 12 pg/mL for BDNF and 31.2 pg/mL for GDNF.

2.16. Statistical analyses

Differences among groups were statistically analyzed using two-way analysis of variance (ANOVA), followed by Tukey's test. All analyses were performed using the GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA). Values of $P < 0.05$ were considered significant (*a*

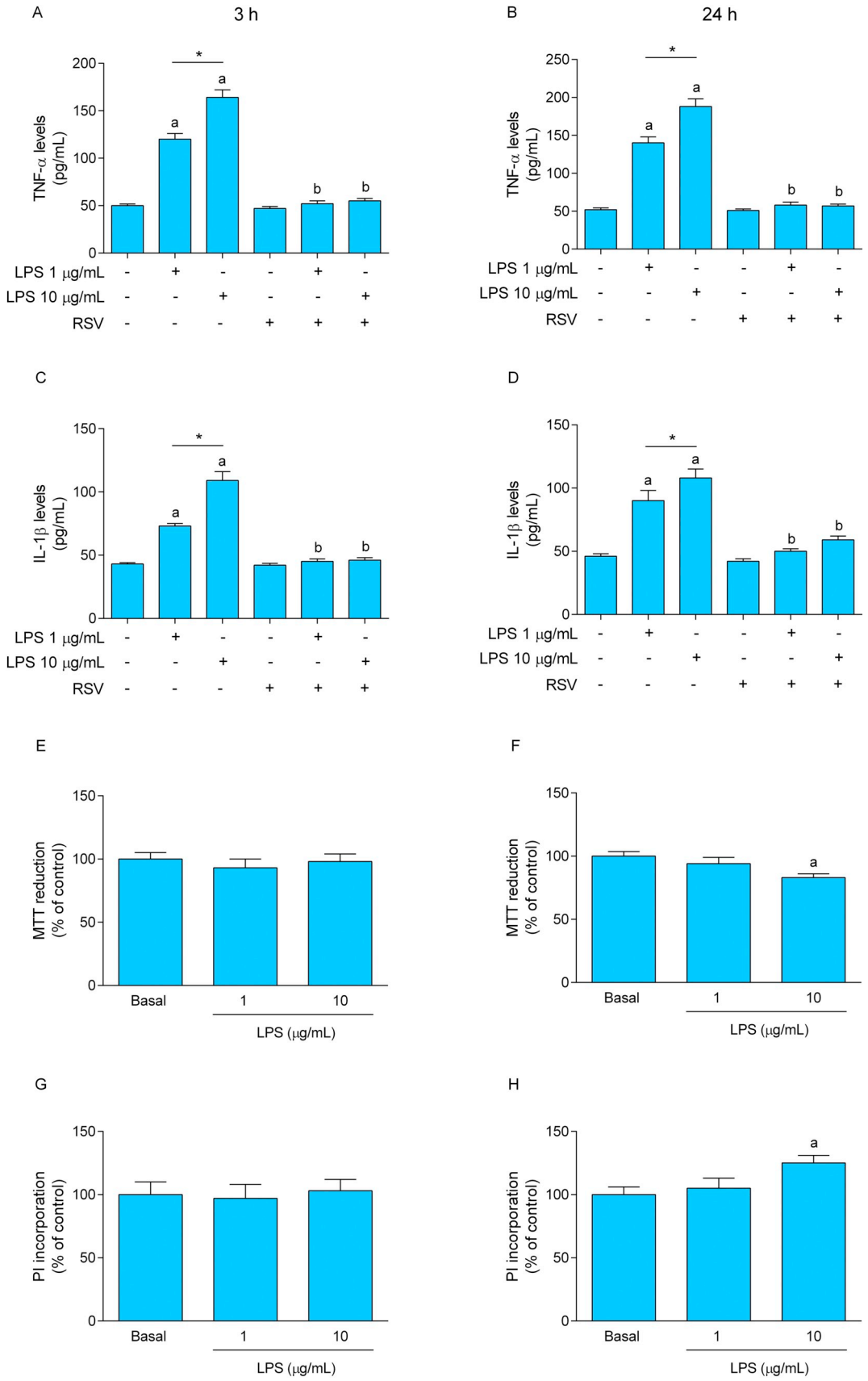

Fig. 1. Effects of LPS and resveratrol on inflammatory response in primary astrocyte cultures. The cells were incubated with 1 or 10 μg/mL LPS for 3 or 24 h, in the presence or absence of resveratrol (100 μM). The extracellular levels of TNF-α (A, B) and IL-1β (B, C), MTT reduction (E, F) and PI incorporation (G, H) were evaluated. The data represent the means ± S.E.M. of at least three independent experiments performed in triplicate. Differences between groups were statistically analyzed using one or two-way ANOVA, followed by Tukey's test. *P* values < 0.05 were considered significant. *a* indicates differences from basal conditions; * indicates difference between LPS doses.

refers to statistically significant differences from the control; *b* refers to statistically significant differences from LPS stimulation).

3. Results

3.1. Resveratrol prevented LPS-induced inflammatory response through adenosine receptors

To ensure that LPS triggered an inflammatory response, we evaluated the levels of pro-inflammatory cytokines in the extracellular medium from primary astrocyte cultures after 3 or 24 h of LPS (1 and 10 μg/mL) challenge. As expected, LPS induced a dose-dependently increase in TNF-α and IL-1β at 3 h (Fig. 1A, C, respectively) and this augment was potentiated after 24 h (Fig. 1B, D).

To test the glial anti-inflammatory activity of resveratrol, we pretreated cultured astrocytes with resveratrol (100 μM) prior LPS incubation. Fig. 1 also displays that resveratrol significantly prevented LPS-induced inflammatory response, maintaining TNF-α and IL-1β levels near to basal values both after 3 and 24 h (Fig. 1A–D). Resveratrol *per se* had no effect on cytokine release.

Then, we measured the cell viability and membrane integrity in this experimental *in vitro* model and observed that LPS did not change these parameters after 3 h of incubation (Fig. 1E, G). However, the highest LPS concentration tested (10 μg/mL) slightly decreased cell viability (17%, Fig. 1F) and increased membrane permeability (25%, Fig. 1H) at 24 h. After these results, we conducted further experiments with LPS 1 μg/mL for 24 h, because these experimental conditions efficiently triggered an inflammatory response, but did not impair cellular membrane integrity.

Subsequently, we considered a potential involvement of adenosine receptors as potential targets in the anti-inflammatory effect of resveratrol. Thus, we co-incubated selective adenosine receptor antagonists concomitantly with resveratrol for 1 h prior the addition of LPS for 24 h. When astrocytes were incubated with the A_1 receptor antagonist DPCPX or A_3 receptor antagonist MRS 1220, the protective effects of resveratrol against LPS-induced TNF-α and IL-1β release were completely abolished (Fig. 2A, B). Moreover, ZM 241385, an A_{2A} antagonist, was also able to prevent the effect of resveratrol, but only on IL-1β release (Fig. 2B). It is important to note that the inhibitors did not change LPS-induced increase in pro-inflammatory cytokines and had no effect *per se* (data not shown). Consistent with an involvement of adenosine receptors in the anti-inflammatory activity of resveratrol, 100 μM caffeine – a non-selective receptor antagonist – also blocked the effect of resveratrol on LPS-induced TNF-α and IL-1β release (Fig. 2A, B). Caffeine alone did not change cytokine release. In addition, similar effects were observed with a higher concentration of caffeine (1000 μM) as well as when it was incubated for 3 h in the presence of LPS and/or resveratrol (Table S1).

We also analyzed the TNF-α and IL-1β mRNA expression. In agreement with our results on cytokine release, LPS induced an increase of mRNA levels of both TNF-α and IL-1β, while resveratrol prevented this stimulatory effect, but not in the presence of caffeine (Fig. 2C, D).

To ensure the involvement of adenosine receptors in the glial anti-inflammatory effect of resveratrol, we evaluated the expression of adenosine receptors. LPS decreased mRNA levels of A_1, A_{2A}, and A_3 (Fig. 3A, B, D respectively). In contrast, resveratrol markedly increased the expression of the same receptors, as well as prevented the effect of LPS. The expression of the adenosine receptor A_{2B} was not changed neither LPS nor resveratrol (Fig. 3C).

3.2. Caffeine abolished the effect of resveratrol in the release of other cytokines after LPS challenge

In order to further describe the role of adenosine receptors in the

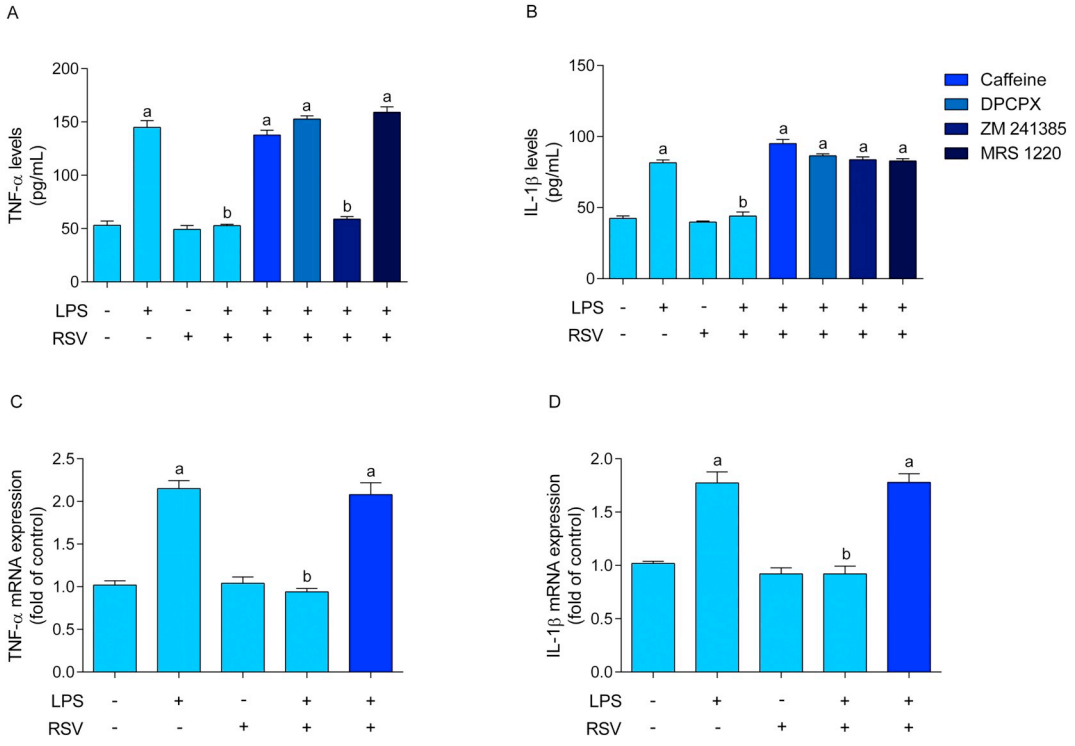

Fig. 2. Adenosine receptor antagonists abolished the anti-inflammatory effect of resveratrol. The cells were co-incubated with 100 μM resveratrol and/or caffeine (non-selective adenosine receptor antagonist; 100 μM), DPCPX (A_1 antagonist; 100 nM), ZM 241385 (A_{2A} antagonist; 50 nM), MRS 1220 (A_3 antagonist; 1 μM) for 1 h, followed by the addition of LPS (1 μg/mL) for 24 h. TNF-α and IL-1β release (A and B, respectively), as well as TNF-α and IL-1β mRNA expression (C and D, respectively) were measured. The data represent the means ± S.E.M. of at least three independent experiments performed in triplicate. Differences between groups were statistically analyzed using two-way ANOVA, followed by Tukey's test. *P* values < 0.05 were considered significant. *a* indicates differences from basal conditions; *b* indicates differences from LPS stimulation.

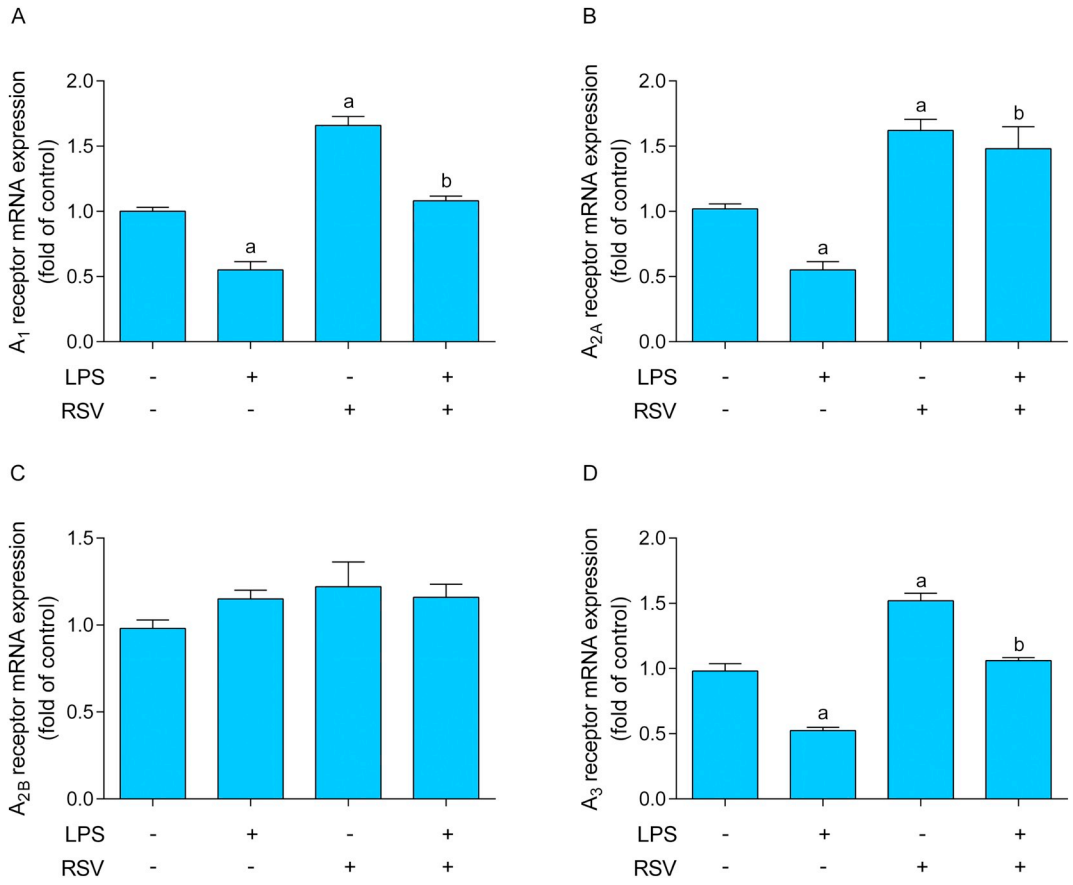

Fig. 3. Effects of LPS and resveratrol on adenosine receptors mRNA expression. The cells were pre-incubated with 100 μM resveratrol, then LPS was added (1 μg/mL) for 24 h. The mRNA encoding A_1, A_{2A}, A_{2B}, and A_3 adenosine receptors subtypes (A–D, respectively) were quantified by RT-PCR. The data represent the means ± S.E.M. of at least three independent experiments performed in triplicate. Differences between groups were statistically analyzed using two-way ANOVA, followed by Tukey's test. *P* values < 0.05 were considered significant. *a* indicates differences from basal conditions; *b* indicates differences from LPS stimulation.

glioprotective effect of resveratrol against neuroinflammation, we assessed the release of other inflammatory mediators. LPS increased the extracellular levels of IL-6 (Fig. 4A), IL-18 (Fig. 4B) and MCP-1 (Fig. 4C), but decreased the release of anti-inflammatory cytokine IL-10 (Fig. 4D). Resveratrol completely prevented these effects and it was able to increase IL-10 levels *per se*. In all these parameters, co-incubation with caffeine abolished the effect of resveratrol on inflammatory response. In addition, the TNF-α/IL-10 and IL-1β/IL-10 ratios were calculated as a measure of the balance between pro- and anti-inflammatory mediators release (Fig. 4E, F, respectively).

3.3. Signaling mechanisms underlying the glioprotective effect of resveratrol against inflammation

To elucidate the signaling mechanisms associated to inflammation, we measured the expression or transcriptional activity of classical inflammatory pathways. Fig. 5 illustrates that LPS increased the mRNA expression levels of NFκB, the master regulator of inflammatory response, as well as the nuclear levels of p65 NFκB (Fig. 5A, B). The protein levels of p38 MAPK, an upstream regulator of NFκB transcriptional activity, were also increased in response to LPS (Fig. 5C). Supporting its glioprotective effect during a pro-inflammatory stimulus, resveratrol prevented the increase of both NFκB p65 and p38 levels. However, caffeine abolished the effectiveness of resveratrol. Considering the crucial role of NFκB and p38 MAPK in triggering inflammatory responses, we evaluated the action of selective antagonists of A_1 (DPCPX), A_{2A} (ZM 241385), and A_3 (MRS 1220) receptors on NFκB transcriptional activity and p38 MAPK levels. In accordance with the results using caffeine, selective adenosine receptor antagonists blocked the effects of resveratrol (Fig. 5A, B).

Subsequently, we demonstrated that LPS increased the mRNA levels of the enzyme COX-2, and resveratrol protected astrocytes from this effect (Fig. 5D). In addition, TLR2 and TLR4, which respond to a wide variety of inflammogens, were also upregulated by LPS, whereas these effects were avoided in the presence of resveratrol (Fig. 5E, F). However, when caffeine was co-incubated, resveratrol was no longer able to maintain the expression of COX-2, TLR2 and TLR4 at basal levels.

3.4. Antioxidant effect of resveratrol was not dependent of adenosine receptors

Inflammatory response is also commonly associated with overproduction of superoxide and nitric oxide, mainly as consequence of NOX activity and iNOS upregulation, respectively. Astrocytes treated with LPS showed a higher NOX activity compared to control conditions (Fig. 6A), which was accompanied by an increase in superoxide levels (Fig. 6B). Additionally, LPS promoted an upregulation in iNOS mRNA expression levels (Fig. 6C). Resveratrol prevented LPS-induced oxidative/nitrosative stress, but had no effect *per se*. Conversely, caffeine did not suppress the antioxidant effect of resveratrol, indicating a major role of adenosine receptors in the anti-inflammatory activity of resveratrol.

3.5. Adenosine receptor antagonists blocked the resveratrol-induced increase in trophic factor release

We also measured the release of BDNF and GDNF from cultured astrocytes in response to LPS and/or resveratrol. LPS significantly

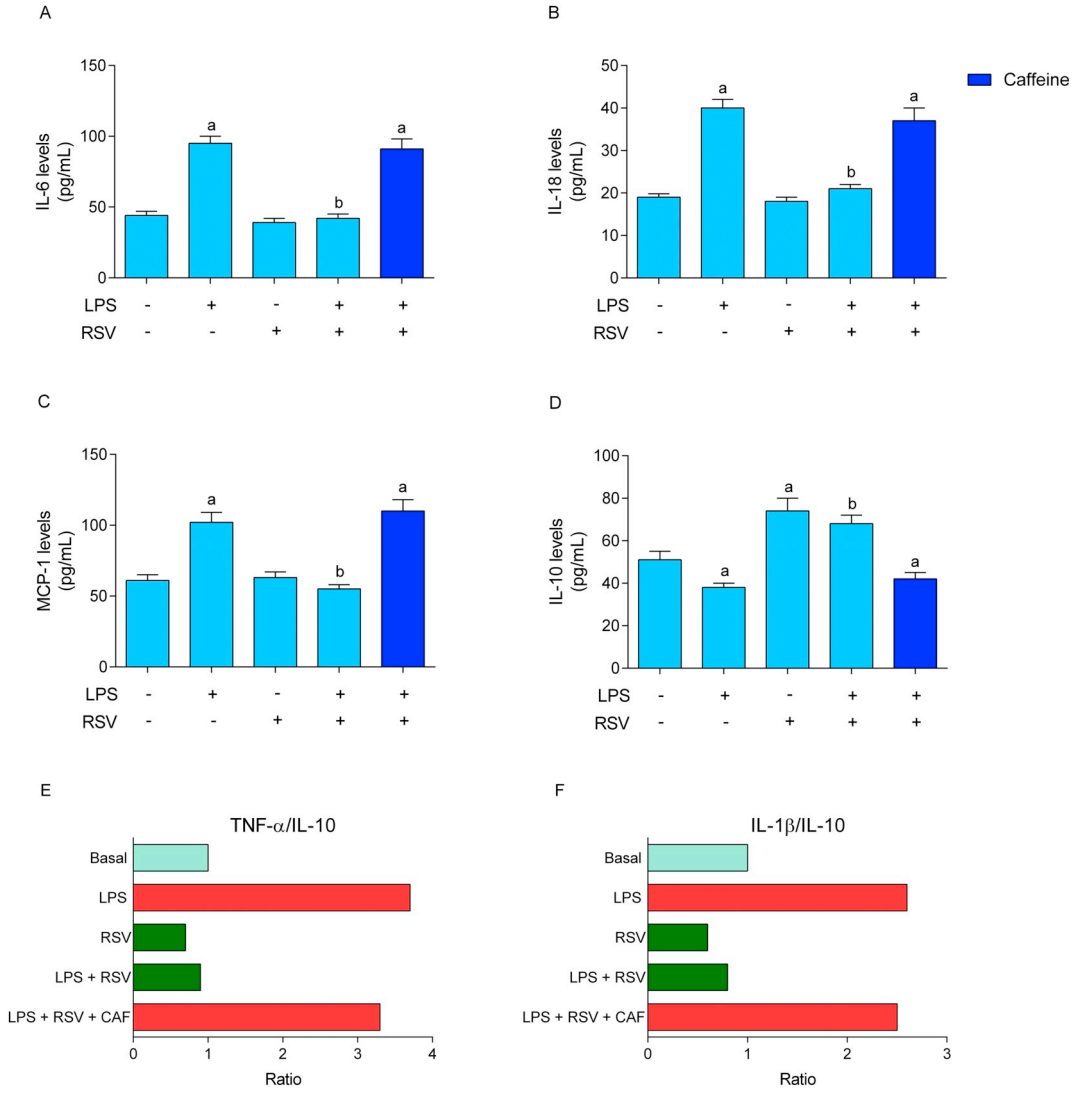

Fig. 4. Caffeine abolished the effect of resveratrol in the release of cytokines after LPS challenge. The cells were co-incubated with 100 μM resveratrol and/or caffeine (non-selective adenosine receptor antagonist; 100 μM) for 1 h, followed by the addition of LPS (1μg/mL) for 24 h. The release of IL-6 (A), IL-18 (B), MCP-1 (C), and IL-10 (D) were measured. The data represent the means ± S.E.M. of at least three independent experiments performed in triplicate. Differences between groups were statistically analyzed using two-way ANOVA, followed by Tukey's test. *P* values < 0.05 were considered significant. *a* indicates differences from basal conditions; *b* indicates differences from LPS stimulation. Additionally, TNF-α/IL-10 (E) and IL-1β/IL-10 (F) ratios were calculated.

decreased the levels of both trophic factors (Fig. 7A, B). Resveratrol prevented the LPS-induced decrease in BDNF and GDNF. Additionally, resveratrol *per se* increased GDNF release and caffeine blocked all effects of resveratrol.

Similarly, all the selective antagonists tested (DPCPX, ZM 241385, and MRS 1220) abolished the action of resveratrol on BDNF and GDNF secretion (Fig. 7A, B), clearly indicating a role of adenosine receptors as important transducers of the glioprotective effects of resveratrol, by modulating trophic signals from astrocytes.

3.6. Resveratrol activated classical protective signaling pathways in astrocytes

Finally, we assessed other pathways related to protective effects of resveratrol. The mRNA expression of SIRT1 (Fig. 8A), Nrf2 (Fig. 8B), HO-1 (Fig. 8C), PI3K (Fig. 8E) and Akt (Fig. 8G), were markedly activated by resveratrol. The inflammatory stimulus with LPS induced a decrease in the mRNA levels of Nrf2/HO-1 and PI3K/Akt, but did not change SIRT1 expression. Under LPS challenge, resveratrol supported a glioprotective effect maintaining mRNA expression levels of HO-1 and PI3K/Akt near to basal values, and increasing the gene expression of Nrf2. Moreover, the co-incubation with caffeine affected only the effect of resveratrol on PI3K/Akt pathway. Thus, we further investigated the phosphorylated forms of PI3K (Fig. 8F) and Akt (Fig. 8H) to confirm the activation of such pathways in the glioprotective effects of resveratrol. In a similar way, LPS decreased phospho-PI3K and phospho-Akt levels. Resveratrol alone increased both of them, and during LPS challenging maintained PI3K/Akt activation near to control levels, but not in the presence of caffeine. However, the protein levels of HO-1 (Fig. 8D) were not significantly changed.

4. Discussion

Astrocytes actively participate in inflammatory response, become an important source of diverse inflammatory mediators, which will impact on the surrounding environment [4,6,13]. Upon LPS challenge, astrocytes presented a pro-inflammatory balance that can be orchestrated by NFκB and its upstream signaling pathway, p38 MAPK, which induce inflammatory gene expression in astrocytes [10,12,43]. Resveratrol markedly prevented the inflammatory response in astrocytes

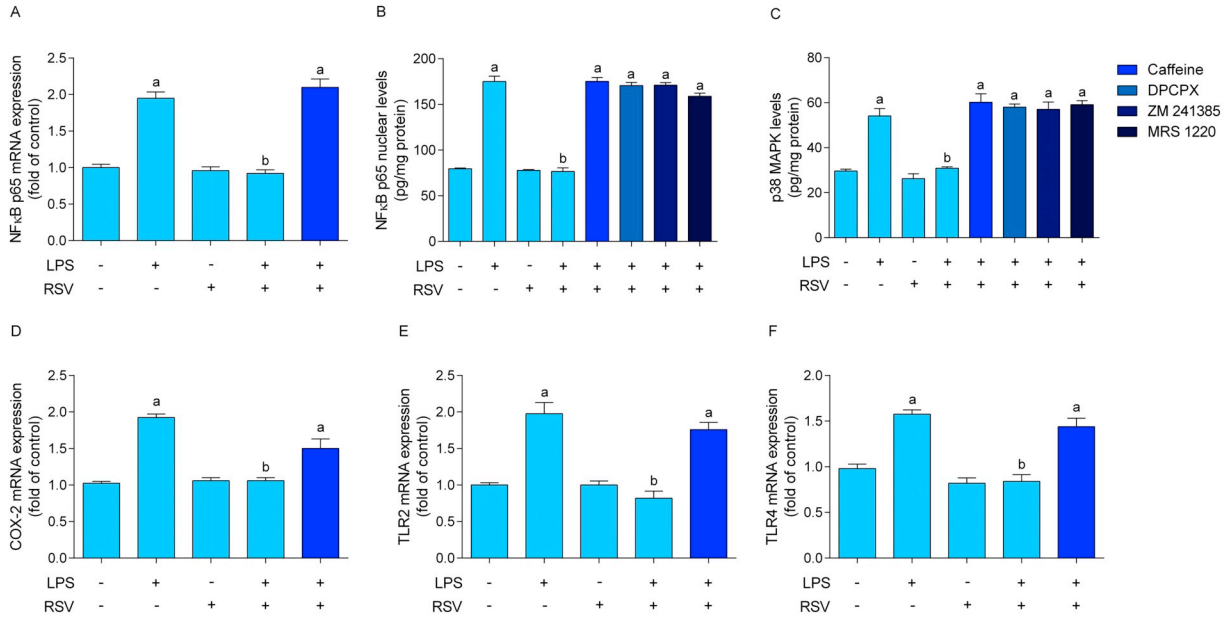

Fig. 5. Resveratrol prevents LPS-induced activation of pro-inflammatory signaling pathways in a mechanism dependent on adenosine receptors. The cells were co-incubated with 100 μM resveratrol and/or caffeine (non-selective adenosine receptor antagonist; 100 μM), DPCPX (A_1 antagonist; 100 nM), ZM 241385 (A_{2A} antagonist; 50 nM), MRS 1220 (A_3 antagonist; 1 μM) for 1 h, followed by the addition of LPS (1 μg/mL) for 24 h. The mRNA expression levels (A) and nuclear content (B) of NFκB, protein levels of p38 MAPK (C), and mRNA levels of the enzyme COX-2 (D), TLR2 (E) and TLR4 (F) were evaluated. The data represent the means ± S.E.M. of at least three independent experiments performed in triplicate. Differences between groups were statistically analyzed using two-way ANOVA, followed by Tukey's test. *P* values < 0.05 were considered significant. *a* indicates differences from basal conditions; *b* indicates differences from LPS stimulation.

challenged with LPS through adenosine receptors and modulation of NFκB and p38. Additionally, resveratrol could protect astrocytes from oxidative/nitrosative damage and activated several defensive signaling pathways, including Nrf-2/HO-1, SIRT1 and PI3K/Akt. These data are in accordance with previous studies that have reported the anti-inflammatory actions of resveratrol in glial cells [7,20,44–48]. However, the identification of new cellular targets of resveratrol is important to further understand the mechanisms by which this compound mediates its neuroimmunomodulatory functions. In this study, we described for the first time the involvement of adenosine receptors in the glial anti-inflammatory activity of resveratrol (Fig. 9). In this regard, our results can contribute for understanding molecular mechanisms involved in resveratrol-mediated protective effects in the CNS, such as in depression and other affective/psychiatric disorders [19,49,50], and neurodegenerative diseases [14,51,52], focused on glial cells.

Adenosinergic system has been involved in some biological activities of resveratrol [22–25], but the dynamics of adenosine receptor expression in astrocytes after resveratrol exposure, and its consequences during inflammatory conditions deserve further investigation. Adenosine receptors comprise four distinct G-protein-coupled subtypes (A_1, A_{2A}, A_{2B} and A_3). A_1 and A_3 receptors are coupled to G_i protein signaling, decreasing intracellular cyclic *adenosine monophosphate* (cAMP) levels, while A_{2A} and A_{2B} subtypes increase cAMP signaling because they are coupled to G_s protein [26,53]. The expression pattern of the subtypes thus orchestrates the cellular responses. Previous studies have demonstrated that activation of adenosine receptors in immune cells can suppress signaling pathways that induce the production of pro-inflammatory mediators, such as TNF-α [32,33,35], in addition to enhance the production of the anti-inflammatory IL-10 [54].

Here, we reported that LPS and resveratrol differentially modulated the astrocytic expression of adenosine receptors. LPS stimulation decreased the mRNA levels of A_1, A_{2A}, and A_3, while resveratrol was able to avoid the effect of LPS, in addition to upregulate the same receptors in unstimulated astrocytes. In agreement with the involvement of adenosine receptors in preventing inflammatory response, when primary astrocyte cultures were co-incubated with their selective antagonists, resveratrol no longer prevented the increase of TNF-α and IL-1β levels promoted by LPS. Caffeine, a non-selective inhibitor of adenosine receptors [10,55,56], reproduced this effect. Therefore, these results suggest that the anti-inflammatory effect of resveratrol occurred *via* an adenosine receptor-dependent mechanism. Interestingly, three subtypes of adenosine receptors similarly affected the anti-inflammatory activity of resveratrol, regardless of the different intracellular signaling triggered by them. In accordance with our results, A_1 and A_3 activation reduced LPS-induced inflammation in astrocytes and microglial cells [31,57], and A_{2A} stimulation could also attenuate inflammatory responses [58,59]. Although A_{2B} receptors can counteract neuroinflammation [34,60], in our study, they were not modulated by neither resveratrol nor LPS.

Previous studies reported that adenosine receptors self-regulate their activation and/or expression, *e.g.* A_{2A}-mediated inhibitory signaling may be effectively counteracted by A_3 activation, while a decrease in A_3 expression shifted the balance toward A_{2A} signaling in microglial cells [59]. Recent evidence has also demonstrated the ability of resveratrol to modulate adenosinergic system in the aging brain, including adenosine receptor gene expression and downstream transduction pathways, as well as enzymes involved on adenosine metabolism [25]. It is important to note that adenosine-mediated signaling may play a relevant role in other neuroprotective effects of resveratrol. Adenosinergic system could control glutamate homeostasis [61] and excitotoxicity in neurodegeneration [29,62], and our group has been widely demonstrated the actions of resveratrol on glutamate uptake and release from astrocytes [38,63].

Several extracellular factors and intracellular signaling pathways govern astrocyte-mediated inflammatory responses. TNF-α acts as a first signal that enhances the production of other pro-inflammatory cytokines such as IL-1β, which in turn has been shown to play a role in apoptosis and blood–brain barrier disruption [6,64]. Consequently, secondary waves of immune cell infiltration into the CNS can occur, leading to neuronal death. Therefore, the ability of resveratrol to avoid the exacerbation of inflammatory processes is crucial for its protective effects in the CNS. Interestingly, we observed that A_1, A_{2A}, and A_3

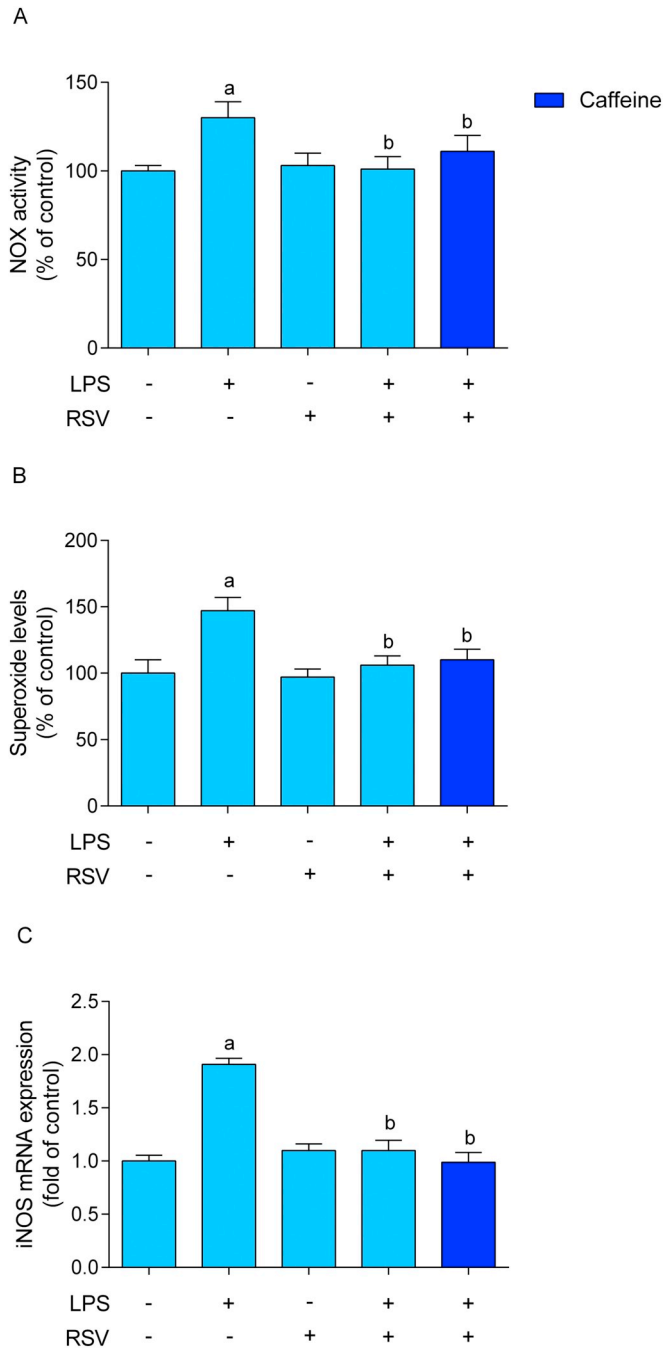

Fig. 6. Antioxidant effect of resveratrol in LPS-challenged astrocytes was not affected by caffeine. The cells were co-incubated with 100 μM resveratrol and/or caffeine (non-selective adenosine receptor antagonist; 100 μM) for 1 h, followed by the addition of LPS (1 μg/mL) for 24 h. NOX activity (A), superoxide levels (B), and iNOS mRNA expression (C) were assessed. The data represent the means ± S.E.M. of at least three independent experiments performed in triplicate. Differences between groups were statistically analyzed using two-way ANOVA, followed by Tukey's test. *P* values < 0.05 were considered significant. *a* indicates differences from basal conditions; *b* indicates differences from LPS stimulation.

receptors participated in the effect of resveratrol in preventing LPS-induced IL-1β release. Similarly, the effect of resveratrol on TNF-α release was dependent on A_1 and A_3 receptors, but did not involve A_{2A} subtype. These results suggest different mechanisms for TNF-α and IL-1β induction and/or repression in astrocytes, and different neurotransmitter and neuromodulator systems have been demonstrated to be

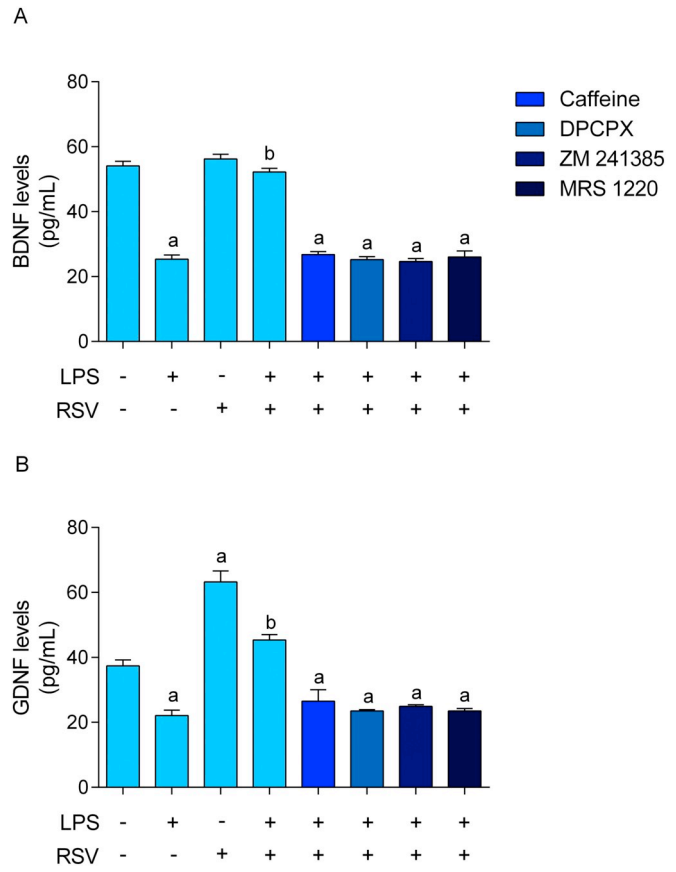

Fig. 7. The glioprotective effect of resveratrol in trophic factor release was blocked by adenosine receptor antagonists. The cells were co-incubated with 100 μM resveratrol and/or caffeine (non-selective adenosine receptor antagonist; 100 μM), DPCPX (A_1 antagonist; 100 nM), ZM 241385 (A_{2A} antagonist; 50 nM), MRS 1220 (A_3 antagonist; 1 μM) for 1 h, followed by the addition of LPS (1 μg/mL) for 24 h. BDNF (A) and GDNF (B) levels were measured in the extracellular medium. The data represent the means ± S.E.M. of at least three independent experiments performed in triplicate. Differences between groups were statistically analyzed using two-way ANOVA, followed by Tukey's test. *P* values < 0.05 were considered significant. *a* indicates differences from basal conditions; *b* indicates differences from LPS stimulation.

able to regulate neuro-immune communication [65].

In addition to TNF-α and IL-1β, we evaluated other cytokines and signaling pathways as effector mechanisms of LPS-induced neuroinflammation, and as potentially targets of resveratrol anti-inflammatory effects. LPS evoked an increase in IL-6, IL-18 and MCP-1, as well as a decrease in IL-10 levels. IL-6 can be produced by astrocytes and microglia, and its levels are greatly increased during brain injuries, being a key player in neuroinflammation [66]. IL-18, a member of IL-1 superfamily, also induces inflammatory processes and is directly associated to neurodegenerative diseases [67]. The chemokine MCP-1, released by astrocytes in response to cytokines, mediates the attraction of microglia and peripheral immune cells to the brain, including monocytes, basophils, T and B cells, thus potentiating the neuroinflammation [6,68]. Conversely, IL-10 displays an anti-inflammatory role, which is mainly related to inhibition of TNF-α secretion, in addition to exert neuroprotection [69,70]. Resveratrol was able to prevent the LPS-induced shift toward a pro-inflammatory balance, and in unstimulated astrocytes, promoted an anti-inflammatory phenotype. Importantly, the effects of resveratrol were abolished in the presence of caffeine, suggesting the involvement of adenosine receptors in such processes.

NFκB is considered the major transcription factor for the regulation of cytokine expression, sustaining the vicious cycle of inflammatory response [43,71]. A wide range of signaling pathways can promote the

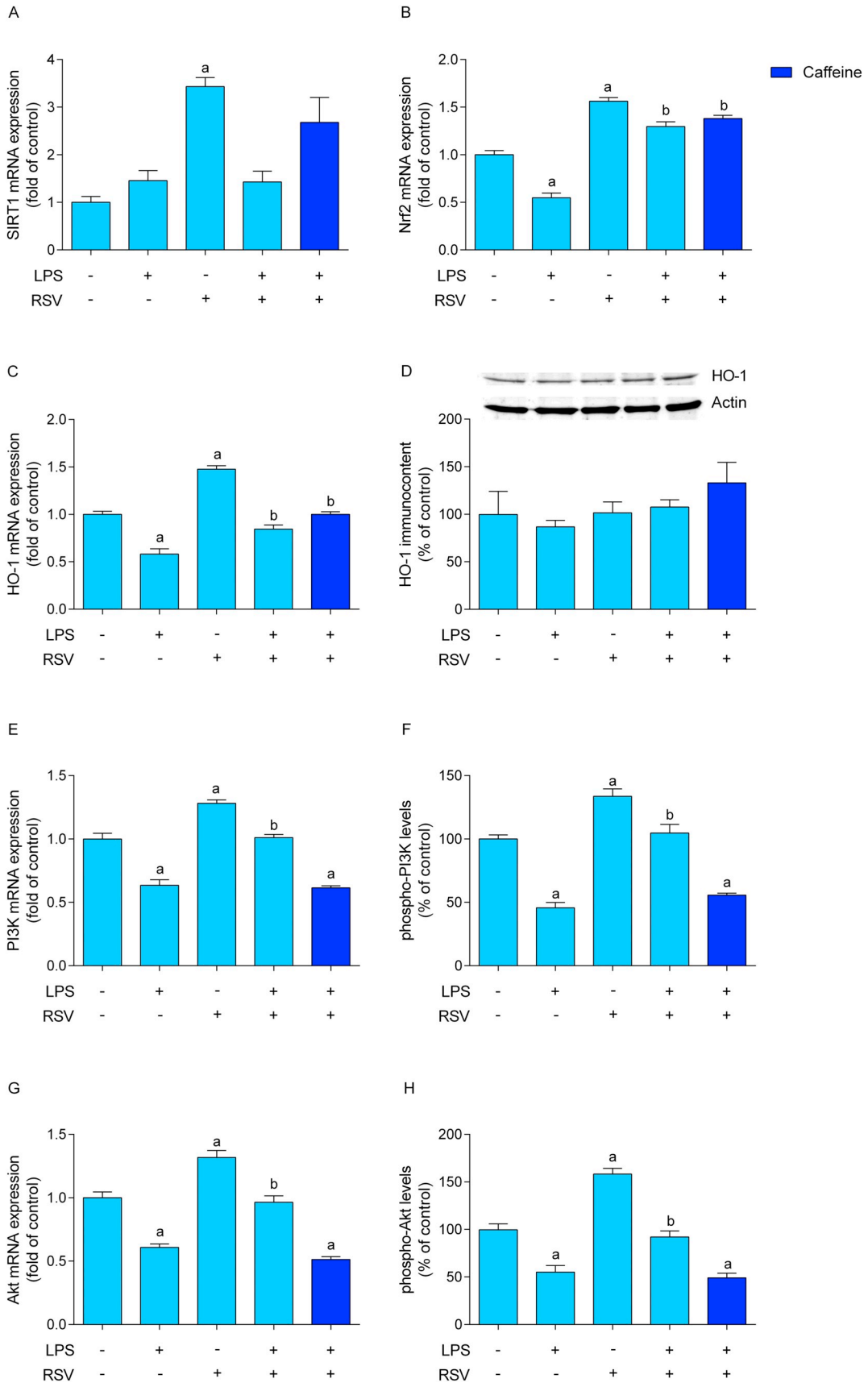

Fig. 8. Resveratrol activated classical signaling pathways associated to its protective effects. The cells were co-incubated with 100 μM resveratrol and/or caffeine (non-selective adenosine receptor antagonist; 100 μM) for 1 h, followed by the addition of LPS (1 μg/mL) for 24 h. The mRNA expression of SIRT1 (A), Nrf2 (B), HO-1 (C), PI3K (E) and Akt (G) were evaluated, as well as the protein levels of HO-1 (D), phospho-PI3K (F) and phospho-Akt (H). The data represent the means ± S.E.M. of at least three independent experiments performed in triplicate. Differences between groups were statistically analyzed using two-way ANOVA, followed by Tukey's test. *P* values < 0.05 were considered significant. *a* indicates differences from basal conditions; *b* indicates differences from LPS stimulation.

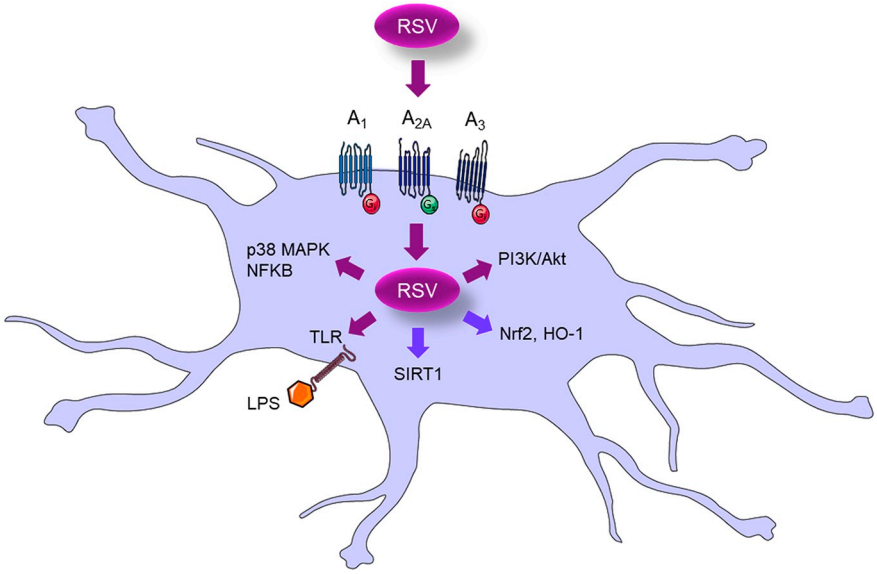

Fig. 9. Schematic illustration of cellular targets of resveratrol (RSV) glioprotection. Our results show that resveratrol was able to modulate the expression of adenosine receptors. Additionally, using selective antagonists and caffeine, we showed that A_1, A_{2A}, and A_3 receptors are involved in the anti-inflammatory effects of resveratrol challenged with LPS. Resveratrol was also able to modulate the expression or activation of important signaling pathways, including PI3K/Akt, Nrf2, HO-1, SIRT1, TLR receptors, p38 MAPK and NFκB. Adenosine receptor-dependent glioprotective effects of resveratrol are showed in purple arrows. Other effects of resveratrol, independent on adenosine receptors, are indicated by blue arrows.

NFκB activation and nuclear translocation, including p38 MAPK. The MAPK superfamily is a critical component that coordinates incoming signals generated by a variety of extracellular stimuli, transmitting the signal down the cascade through the phosphorylation of regulatory proteins, including transcription factors [72]. LPS classically stimulates the p38 MAPK/NFκB pathway to induce the inflammatory response [12], while the attenuation of this signaling by resveratrol contributes to its anti-inflammatory effect [7,16], in accordance with our data.

We showed that selective antagonists of A_1, A_{2A}, and A_3 receptors, as well as caffeine, blocked the actions of resveratrol, suggesting that adenosine receptors can be an upstream effector by which resveratrol controls p38 MAPK/NFκB activation. Supporting this hypothesis, previous studies have shown that activation of adenosine receptors can decrease the downstream phosphorylation of p38 MAPK in different experimental cell models [73,74]. Moreover, to our knowledge, our study provides the first evidence that adenosine receptors are potentially related to the suppression of NFκB activation by resveratrol in astrocytes. Furthermore, activation of A_{2A} receptor subtype can inhibit TLR-induced cytokine responses [59]. Here, we also reported that resveratrol prevented TLR2 and TLR4 gene upregulation caused by LPS, but not when co-incubated with caffeine. Importantly, TLRs are major activators of p38 MAPK/NFκB pathway, thus resveratrol potentially attenuates astrocyte activation in an inflamed milieu with the participation of adenosine receptor signaling.

Inflammatory responses also involve the overproduction of superoxide and nitric oxide, though NOX and iNOS activation, respectively [75,76]. As consequence, severe oxidative/nitrosative damage and neural death can occur [77,78]. We observed that resveratrol was able to prevent the increase in superoxide production, NOX activity and iNOS expression induced by LPS in astrocytes. However, caffeine did not modulate resveratrol effects, indicating that other mechanisms, rather than adenosine receptors, are primary responsible for the antioxidant activity of resveratrol in astrocytes. Among them, Nrf2 regulates many antioxidant genes, such as HO-1 [79]. Nrf2/HO-1 pathway has been proposed as an important mechanism by which resveratrol exerts glioprotection [7,17], independently of adenosine receptors. Moreover, astrocytes have been showed as the predominant cell type for activation of Nrf2 [80], indicating these cells as important targets to protective and/or therapeutic strategies. Here, we observed that both LPS and resveratrol on HO-1 gene induction, particularly at the transcriptional level, since HO-1 protein content did not change. However, we cannot rule out alterations in HO-1 protein levels throughout the 24 h of treatment, because different mechanisms are involved in HO-1 protein turnover [81].

Nrf2/HO-1pathway also participate in the anti-inflammatory effects of resveratrol [7]. In addition, other mechanisms have been attributed to resveratrol for counteracting inflammatory response, including induction of SIRT1 [46], suppressor of cytokine signaling (SOCS) proteins [47], adenosine monophosphate-activated protein kinase (AMPK) [21], and mammalian target of rapamycin (mTOR) signaling pathways [48]. In the present study, we reinforced the role of SIRT1 and PI3K/Akt pathway, classic pathways that are associated with the biological effects of resveratrol. PI3K/Akt pathway can negatively regulate LPS signaling by inhibiting the activation of MAPKs, while inhibition of PI3K presents an opposite effect, enhancing inflammation [82]. In primary astrocyte cultures, we found that LPS decreased PI3K and Akt gene expression and activation, which were prevented by resveratrol. Additionally, resveratrol stimulated an increase of both PI3K and Akt mRNA levels and phosphorylated forms in the absence of LPS. Both of these effects of resveratrol were abolished in the presence of caffeine, showing the role of adenosine receptors for induction of PI3K and Akt. Importantly, adenosine receptors stimulate PI3K/Akt pathway in different cell types [82–85]. Therefore, the cross talk between PI3K/Akt and MAPK signaling pathways, and the modulation by adenosine receptors, may play a crucial role in the neuroimmunomodulatory and glioprotective functions of resveratrol.

Astrocytes also are well known to execute a neurotrophic role by releasing trophic factors such as BDNF and GDNF [86]. In addition to modulate the inflammatory response, resveratrol avoided the decrease in the secretion of BDNF and GDNF from astrocytes in response to LPS. Resveratrol *per se* also increased GDNF extracellular levels. Selective antagonists of adenosine receptors, as well as caffeine, abolished all these protective effects. Previous studies have demonstrated that activation of adenosine receptors can promote the release of BDNF from microglial cells [87] and GDNF expression in astrocytes [88]. Both BDNF and GDNF are essential for neuronal survival and synaptic plasticity, and a decrease of their functions leads to neuronal injury and contribute to neurodegenerative diseases [86]. Importantly, GDNF can inhibit microglial activation, thus playing a role in the control of neuroinflammation [89]. In this regard, the release of trophic factors by astrocytes might represent an important feature of the resveratrol glioprotection.

Interestingly, the incubation of astrocytes with adenosine did not prevent the inflammatory response induced by LPS nor potentiate the effects of resveratrol (data not shown), in accordance with previous reports [10,90]. These data prompt the hypothesis that the modulation

of adenosine receptors by resveratrol is not dependent on the classical agonist, as it has been described for another anti-inflammatory and neuroprotective molecule, paeoniflorin [91]. Additionally, recent evidence suggests possible interactions between resveratrol and adenosine receptors, using computational methods [24]. Thus, the anti-inflammatory effects of resveratrol observed in our study may be due to such interaction. Our data provide important clues for understanding the molecular basis underlying the glioprotective mechanisms played by this polyphenol under inflammatory conditions, and reinforce the potential role of resveratrol as a glial-target agent in CNS disorders that involve neuroinflammation.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbadis.2019.01.004.

Transparency document

The Transparency document associated with this article can be found, in online version.

Acknowledgments

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Declarations of interest

None.

Data statement

All data and figures that support the findings of this study are original and are included within the article. Additionally, they are available, as well as all methods, upon request to the corresponding author Larissa Daniele Bobermin by the e-mail: larissabobermin@gmail.com.

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