Guanosine inhibits LPS-induced pro-inflammatory response and oxidative stress in hippocampal astrocytes through the heme oxygenase-1 pathway

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Abstract Guanosine, a guanine-based purine, is an extracellular signaling molecule that is released from astrocytes and has been shown to promote central nervous system defenses in several in vivo and in vitro injury models. Our group recently demonstrated that guanosine exhibits glioprotective effects in the C6 astroglial cell line by associating the heme oxygenase-1 (HO-1) signaling pathway with protection against azide-induced oxidative stress. Astrocyte overactivation contributes to the triggering of brain inflammation, a condition that is closely related to the development of many neurological disorders. These cells sense and amplify inflammatory signals from microglia and/or initiate the release of inflammatory mediators that are strictly related to transcriptional factors, such as nuclear factor kappa B (NFκB), that are modulated by HO-1. Astrocytes also express toll-like receptors (TLRs); TLRs specifically recognize lipopolysaccharide (LPS), which has been widely used to experimentally study inflammatory response. This study was designed to understand the glioprotective mechanism of guanosine against the inflammatory and oxidative damage induced by LPS exposure in primary cultures of hippocampal astrocytes. Treatment of astrocytes with LPS resulted in deleterious effects, including the augmentation of pro-inflammatory cytokine levels, NFκB activation, mitochondrial dysfunction, increased levels of oxygen/nitrogen species, and decreased levels of antioxidative

defenses. Guanosine was able to prevent these effects, protecting the hippocampal astrocytes against LPS-induced cytotoxicity through activation of the HO-1 pathway. Additionally, the anti-inflammatory effects of guanosine were independent of the adenosinergic system. These results highlight the potential role of guanosine against neuroinflammatory-related diseases.

Keywords Guanosine · Astrocytes · Neuroinflammation · Glioprotection · HO-1 · NFκB

Introduction

Neuroinflammation, well-marked by activation of the innate immune system of the brain, plays a role in the early mechanisms involved in the pathology of many neurodegenerative and acute illnesses, such as Alzheimer's disease, Parkinson's disease, and stroke [1–3]. Brain inflammation is triggered by the activation of glial cells, which can promote an increase in the infiltration of peripheral immune cells, the production of inflammatory mediators, and the generation of reactive oxygen/nitrogen species (ROS/RNS) [4]. Thus, proper regulation of inflammation by glial cell management could be of vital importance to delay disease progression in several neurological disorders.

Astrocytes are the most abundant glial cell type in the central nervous system (CNS), and under physiological conditions, they play key housekeeping roles offering structural, metabolic, and trophic support to neuronal survivor [5–7]. However, astrocyte overactivation promotes the release of a broad array of cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor α (TNF-α), chemokines, and ROS. Secondary waves of immune cell infiltration into the CNS are also a result, and all of these processes may lead to cognitive

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decline and neuronal apoptosis [8, 9]. Although activation of these cells is a key component of several neurodegenerative and/or acute conditions and significantly contributes to behavioral and cognitive deficits, their involvement in neuroinflammatory process has remained largely unexplored.

Lipopolysaccharide (LPS), the main component of the outer membrane of Gram-negative bacteria, has been used as a classical model of innate recognition, which leads to a robust inflammatory response by activation of immunocompetent cells [10, 11]. On the cellular level, LPS induces changes in protein expression, which is triggered by its binding to Toll-like receptor 4 (TLR4), which is present in astrocytes [10, 12]. Exposure to LPS can lead to the translocation of nuclear factor kappa B (NFκB) into the nucleus, inducing the transcription of inflammatory genes, nitric oxide (NO) release, and the overproduction of ROS [10–12]. Therefore, it is likely that activation of NFκB that occurs in astrocytes following neuroinflammation plays a key role in the pathophysiology of the development of CNS disorders.

Guanosine, a guanine-based purine, may be released from astrocytes and has been shown to exhibit neuroprotective effects in several *in vivo* and *in vitro* studies [13–19]. This nucleoside can effectively protect cells against hypoxia [20, 21], cytotoxicity induced by the β-amyloid peptide [22], chronic cerebral hypoperfusion [23], ischemic insult [2, 24–26], and quinolinic-acid-induced seizures [15, 17, 27]. However, no studies have investigated the role of guanosine in astrocyte inflammatory response in primary cultures. Although there is increasing evidence of the protective actions of guanosine in glial cells, the mechanisms of these effects are not fully understood. Recently, some authors described a possible role of the adenosinergic system in guanosine signaling [28]. Previous studies from our group have shown that guanosine possesses important antioxidant properties that may be derived from both its ability to directly scavenge oxidative species and the activation of pathways involved in antioxidant defenses. Additionally, these studies point to the heme oxygenase-1 (HO-1) pathway as a fundamental defense mechanism for cells exposed to oxidant challenges [29, 30].

It has been reported that HO-1 may be a therapeutic target in neurodegenerative diseases and brain infections [31]. HO is the rate-limiting enzyme in the pathway through which the pro-oxidant heme is degraded into the antioxidants biliverdin and bilirubin. HO-1 is the inducible form, and its expression is maintained at low levels in the normal brain, restricted to small groups of scattered cells, including astrocytes [32]. In conditions of oxidative injury and inflammation, the synthesis of this enzyme is increased, which plays an important role in its neuroprotective function. This crucial role of HO-1 in responding to CNS alterations has generated renewed interest in its regulation and function [30, 33, 34]. HO-1 counteracts NO toxicity by inhibiting inducible nitric oxide synthase (iNOS) activity [35], and NO can interact with superoxide

anions (generated by mitochondria), which leads to overproduction of the powerful oxidant species peroxynitrite [36].

Considering the neuroinflammatory effects of LPS and the well-known role of the hippocampus in brain plasticity as well as the glioprotective functions of guanosine, including its anti-inflammatory and antioxidant activities, the aim of this study was to investigate the effects of guanosine on LPS-induced inflammation in hippocampal primary astrocyte cultures and the putative role of HO-1 in these effects. We expected guanosine to modulate HO-1 pathway, improving antioxidant defenses, preventing NFκB translocation, and consequently decreasing the production of pro-inflammatory cytokines and ROS/RNS. We also aim to better understand the role of adenosine in guanosine glioprotection.

Materials and methods

Chemicals

Dulbecco's modified Eagle's medium/F12 (DMEM/F12), other materials for cell cultures, and NFκB p65 ELISA were purchased from Gibco/Invitrogen (Carlsbad, CA, USA). Guanosine, LPS, 2'-7'-dichlorofluorescein diacetate (DCFH-DA), propidium iodide (PI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) formazan, glutathione (GSH) standard, o-phthalaldehyde, and zinc protoporphyrin IX (ZnPP IX) were obtained from Sigma-Aldrich (St. Louis, MO, USA). TNF-α ELISA was purchased from PeproTech (Rocky Hill, NJ, USA). IL-1β was purchased from eBioscience (USA). All other chemicals were purchased from common commercial suppliers.

Animals

Newborn male Wistar rats were obtained from our breeding colony (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 NIH Guide for the Care and Use of Laboratory Animals and were approved by the Federal University of Rio Grande do Sul Animal Care and Use Committee (process number 27543).

Primary hippocampal astrocyte cultures

This protocol was in accordance with Bellaver et al., 2014 and 2015 [37, 38]. Briefly, the newborn male Wistar rats' hippocampi were aseptically removed from cerebral hemispheres. Tissue was enzymatic (with trypsin 0.05 %) and mechanically dissociated and then centrifuged at 100g for 5 min. Cells were resuspended in Hanks' balanced salt solution (HBSS)

containing DNase (0.003 %) and left for decantation for 20 min. Supernatant was collected and centrifuged for 7 min (400 g). Cells from pellet were resuspended in DMEM/F12 (10 % FBS, 15 mM HEPES, 14.3 mM NaHCO₃, 1 % fungizone, and 0.04 % gentamicin), plated in 6- or 24-well plates pre-coated with poly-L-lysine at a density of $3\text{--}5 \times 10^5$ cells/cm². Cells were cultured at 37 °C in atmosphere with 5 % of CO₂. The first medium exchange occurred 24 h after obtaining the culture. The medium change occurred once every 2 days during the first week and once every 4 days during the second week. Purity of primary astrocyte cultures was assessed by immunocytochemistry for glial fibrillary acidic protein (GFAP); OX-42 and Neu N were used as microglial and neuronal markers, respectively. Under these conditions, cultures were confirmed to contain more than 95 % cells positive to GFAP, indicating the astrocytic phenotype.

Cellular treatments

After the cells reached confluence, the culture medium was exchanged with serum-free DMEM/F12, and cells were pre-incubated in the absence or presence of 100 µM guanosine for 1 h. After this pre-incubation, 10 µg/mL LPS was added for 3 h (guanosine was maintained). During all procedures, the cells were maintained at 37 °C in an atmosphere with 5 % of CO₂. To study the involvement of the HO-1 signaling pathway in the effects of guanosine on LPS-induced inflammatory response, we co-incubated ZnPP IX (10 µM), an HO-1 inhibitor, with guanosine. Additionally, to verify the role of adenosine and caffeine (an adenosine receptor antagonist) on inflammatory response in astrocytes, cells were co-incubated with adenosine (100 and 1000 µM) or guanosine (100 µM) and caffeine (100 and 1000 µM) before the LPS treatments, using the same conditions described above.

TNF- α measurement

Culture medium was collected and the concentration of TNF- α was carried using a rat TNF- α ELISA kit from Peprotech (USA) following the manufacturer's instructions. The results are expressed as the percentage of the control levels.

IL-1 β measurement

IL-1 β was carried out in a culture extracellular medium, using a rat IL-1 β ELISA kit from eBioscience (USA) following the manufacturer's instructions. The results are expressed as the percentage of the control levels.

NF κ B levels

Levels of NF κ B p65 in the nuclear fraction, which had been isolated from lysed cells by centrifugation, were measured using an ELISA commercial kit from Invitrogen (USA). The results are expressed as percentages relative to the control levels.

Cell viability and Membrane integrity

Cell viability was determined using a MTT formazan assay (activity of mitochondrial dehydrogenases). 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 with 5 % of CO₂ [30]. Subsequently, the medium was removed and the MTT crystals were dissolved in dimethylsulfoxide. Absorbance values were measured at 560 and 650 nm. Results are expressed as percentages relative to the control conditions. For PI incorporation assay (membrane integrity), 7.5 µM PI was added, and cells were incubated for 30 min at 37 °C in an atmosphere with 5 % of CO₂ [39]. The optical density of fluorescent nuclei (labeled with PI), used to indicate a loss in membrane integrity, was determined with Optiquant software (Packard Instrument Company). Density values obtained are expressed as a percentage of the control condition.

Mitochondrial membrane potential $\Delta\Psi$ m (JC-1 assay)

To determine the $\Delta\Psi$ m, cells were incubated for 30 min with JC-1 (5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolylcarbocyanine iodide, 2 µg/mL) [40]. Cells were then homogenized and centrifuged, washed once with HBSS, and transferred to a 96-well plate. Fluorescence was measured 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 590 nm (red fluorescent J-aggregates) to 540 nm (green monomers). The results are expressed as percentages relative to the control conditions.

DCFH oxidation

Intracellular ROS levels were detected using DCFH-DA. DCFH-DA was added to the medium at a concentration of 10 µM, and cells were incubated for 30 min at 37 °C. Following DCFH-DA exposure, cells were scraped into phosphate-buffered saline with 0.2 % Triton X-100. The fluorescence was measured in a plate reader (Spectra Max GEMINI XPS, Molecular Devices, USA) with excitation at 485 nm and emission at 520 nm [41]. The results are expressed as percentages relative to the control conditions.

Nitrite levels

NO levels were determined by measuring the amount of nitrite (as oxidation product of NO), as indicated by the Griess reaction. The Griess reagent was prepared by mixing equal volumes of 1 % sulfanilamide in 0.5 M HCl and 0.1 % N-(1-naphthyl) ethylenediamine in de-ionized water. Briefly, the Griess reagent was added directly to the cell culture, which was incubated in the dark for 15 min, at 22 °C [29, 30]. Samples were analyzed at 550 nm on a microplate spectrophotometer. Nitrite concentrations were calculated using a standard curve prepared with concentrations of sodium nitrite ranging from 0 to 50 μ M. The results are expressed as percentages relative to the control conditions.

Superoxide levels

Superoxide levels were determined using the superoxide anion assay kit from Sigma. 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 conditions.

GSH levels

GSH levels were assessed as described previously [42]. Cell 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. GSH concentrations were calculated as nanomoles per milligram of protein. The results are expressed as percentages relative to the control conditions.

Protein determination

Protein content was measured using Lowry's method with bovine serum albumin as a standard [43].

Statistical analyses

Data are presented as mean $\pm$ S.E.M. Each experiment was performed in triplicate from at least four independent cultures. Differences among groups were statistically analyzed using two-way analysis of variance (ANOVA), followed by Tukey's test. Values of $P<0.05$ were considered significant and *a* indicates differences from control conditions, and *b* differences from LPS. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software.

Results

To assess the LPS-induced inflammatory response in astrocytes, the levels of classical pro-inflammatory cytokines were measured. Compared to that found in the control conditions, the release of TNF- α from astrocytes was increased by approximately three times by the addition of LPS (Fig. 1a). LPS also induced a significant increase in IL-1 β levels (90 %) (Fig. 1b). Guanosine significantly prevented these effects, repressing the release of TNF- α and IL-1 β . Guanosine per se did not affect the pro-inflammatory cytokine levels. When cells were incubated with an HO-1 inhibitor (ZnPP IX), all of the effects of guanosine were blocked. Under control conditions, this inhibitor did not change the HO-1 levels (data not shown).

Because some effects of guanosine have been shown to be mediated by adenosinergic system [44–46], the role of adenosine and/or caffeine (adenosine receptor antagonist) in LPS-induced inflammatory response was also evaluated. Interestingly, adenosine (100 and 1000 μ M) did not prevent the increase in cytokine release that occurred after LPS exposure (Table 1). To test whether the effect of guanosine on the inflammatory response was via an adenosine-receptor-dependent mechanism, cells were co-incubated with guanosine and caffeine (100 and 1000 μ M). The presence of caffeine did not change the anti-inflammatory effect of guanosine (Table 1). Adenosine and caffeine per se, at both concentrations, did not significantly affect the levels of TNF- α and IL-1 β (data not shown).

The membrane integrity of hippocampal astrocytes after exposure to LPS and/or guanosine was evaluated by measuring the PI incorporation. Mitochondrial activity is related to the number of viable cells, and it was thus determined by MTT assay. Guanosine and/or LPS did not affect membrane integrity or cell viability (Table 2). Treatment with adenosine (100 and 1000 μ M) and caffeine (100 and 1000 μ M) in the presence or absence of LPS also did not affect PI uptake or MTT reduction (data not shown). Because changes in cell integrity were not observed, the increased levels of cytokines most likely resulted from secretion.

Fig. 1 Guanosine decreases pro-inflammatory cytokine release. Cells were incubated in serum-free DMEM/F12 with 100 μ M guanosine (GUO) for 1 h, followed by the addition of 10 μ g/mL LPS for 3 h. The cells were co-incubated with 10 μ M ZnPP IX along with the guanosine. **a** TNF- α and **b** IL-1 β levels were measured as described in the “Materials and methods” section. Data represent the mean $\pm$ S.E.M. of four

independent experiments that were performed in triplicate. Differences between groups were statistically analyzed using two-way ANOVA, followed by Tukey’s test. Values of $P < 0.05$ were considered significant. *a* indicates differences from control conditions, and *b* differences from LPS challenge

To elucidate the possible anti-inflammatory mechanism of guanosine, NF κ B activation was assessed (Fig. 2). LPS exposure increased NF κ B p65 nuclear levels by 70 % in hippocampal astrocytes. Pre-treatment with guanosine was able to decrease the NF κ B levels significantly (58 %). This effect was dependent of HO-1, which is upstream of NF κ B. Guanosine alone did not affect NF κ B activation.

ROS production plays a critical role in inflammatory response. Mitochondria are the primary site of ROS production. Considering that mitochondrial activity depends on the maintenance of its membrane potential, mitochondrial membrane potential ($\Delta\Psi$ m) dysfunction was evaluated. Figure 3a shows a significant decrease in $\Delta\Psi$ m following LPS exposure (27 %). Guanosine prevented this reduction. In the presence of the HO-1 inhibitor, guanosine no longer prevented the

$\Delta\Psi$ m decrease induced by LPS. Further, as a consequence of mitochondrial dysfunction, intracellular ROS production was measured using DCFH oxidation (Fig. 3b). Compared to the levels found under control conditions, ROS levels were significantly increased by LPS (53 %). Guanosine prevented this effect, decreasing ROS levels from 153 to 108 %. In addition, superoxide production was measured (Fig. 3c). LPS increased cellular superoxide levels by approximately 45 %, and this effect was also prevented by guanosine. The guanosine effect was abolished in the presence of the HO-1 inhibitor. Guanosine had no effect in the control condition. NO production was measured by the formation of nitrite (Fig. 3d). Treatment of the astrocytes with LPS caused a significant increase in NO levels (60 %) compared to control conditions. Guanosine totally inhibited the LPS-induced

Table 1 The effects of guanosine on LPS-induced inflammatory response were independent of the adenosinergic system

Treatment	TNF- α levels (% of control)	IL-1 β levels (% of control)
LPS	280 $\pm$ 18 ^a	190 $\pm$ 12 ^a
GUO+LPS	112 $\pm$ 12 ^b	110 $\pm$ 10 ^b
ADO 100+LPS	271 $\pm$ 15 ^a	182 $\pm$ 12 ^a
ADO 1000+LPS	277 $\pm$ 21 ^a	185 $\pm$ 15 ^a
CAF 100+LPS	283 $\pm$ 22 ^a	180 $\pm$ 14 ^a
CAF 1000+LPS	268 $\pm$ 22 ^a	178 $\pm$ 15 ^a
GUO+CAF 100+LPS	115 $\pm$ 14 ^b	107 $\pm$ 10 ^b
GUO+CAF 1000+LPS	114 $\pm$ 12 ^b	115 $\pm$ 15 ^b

Cells were incubated in serum-free DMEM/F12 with adenosine (100 and 1000 μ M) or 100 μ M guanosine (GUO) and caffeine (100 and 1000 μ M) for 1 h before being treated with 10 μ g/mL LPS for 3 h. TNF- α and IL-1 β levels were measured as described in the “Materials and methods” section. Data represent the mean $\pm$ S.E.M. of three independent experiments that were performed in triplicate. Differences between groups were statistically analyzed using two-way ANOVA, followed by Tukey’s test. Values of $P < 0.05$ were considered significant

^a Differences from control conditions

^b Differences from LPS

Table 2 Guanosine had no effect on cellular viability

Treatment	PI incorporation (% of control)	MTT (% of control)
LPS	95±8	96±8
GUO	100±8	101±8
GUO+LPS	107±8	98±7

Cells were incubated in serum-free DMEM/F12 with 100 μ M guanosine (GUO) for 1 h, followed by the addition of 10 μ g/mL LPS for 3 h. The cells were co-incubated with 10 μ M ZnPP IX along with the guanosine. PI incorporation was measured and MTT assay was conducted as described in the “Materials and methods” section. Data represent the mean±S.E.M. of three independent experiments that were performed in triplicate. Differences between groups were statistically analyzed using two-way ANOVA, followed by Tukey’s test. Values of $P<0.05$ were considered significant

production of NO. In the presence of the HO-1 inhibitor, guanosine did not prevent the overproduction of ROS or NO that was induced in the astrocytes by LPS.

To evaluate antioxidant defenses under conditions of LPS-induced inflammation, GSH content was measured, which is a major source of defense against ROS in astrocytes. As shown in Fig. 4, LPS decreased GSH levels (30 %) when compared to control. Guanosine prevented this effect increasing GSH content from 70 to 98 %. The HO-1 inhibitor abolished the effect of guanosine on GSH content.

Discussion

LPS is able to mediate the induction of pro-inflammatory cytokines and ROS/RNS, which together orchestrate the

Fig. 2 Guanosine prevents LPS-induced NFκB activation. Cells were incubated in serum-free DMEM/F12 with 100 μ M guanosine (GUO) for 1 h, followed by the addition of 10 μ g/mL LPS for 3 h. The cells were co-incubated with 10 μ M ZnPP IX along with the guanosine. NFκB levels were measured as described in the “Materials and methods” section. Data represent the mean±S.E.M. of four independent experiments that were performed in triplicate. Differences between groups were statistically analyzed using two-way ANOVA, followed by Tukey’s test. Values of $P<0.05$ were considered significant. *a* indicates differences from control conditions; and *b*, differences from LPS

pathophysiological events of neuroinflammation. Therefore, downregulation of these mediators, which are produced largely under conditions of disease, would be expected to provide a target for the development of therapeutic approaches against brain inflammation and related diseases, such as neurodegenerative disorders and brain ischemia [2, 47, 48]. In this study, we demonstrated the glioprotective potential of guanosine to prevent inflammatory and oxidative damage caused by LPS in primary cultures of hippocampal astrocytes. We also demonstrated that these effects seem to be mediated via the induction of HO-1, which modulates NFκB activation, a transcription factor that is responsible for the expression of a complex array of injury-responsive genes in the CNS, including iNOS and cytokines such as TNF- α and IL-1 β .

Guanosine has been studied in a variety of experimental neuropathological contexts, including brain trauma [49], glucose deprivation, [29] pathological events involved in spinal cord injury [50], and seizures [14, 27]; however, the precise mechanism of guanosine’s neuroprotective effects is not completely understood. Some data support the existence of specific receptor-like binding sites for guanosine [17, 51, 52], and data also indicate that the extracellular effects of guanosine may involve the activation of intracellular signaling pathways, including the involvement of G proteins, MAPK, PI3K, and HO-1 [17, 30, 51, 53, 54]. Furthermore, receptor-independent mechanisms may mediate effects of guanosine. Guanosine has also been shown to increase the extracellular levels of adenosine [28, 44]. Although adenosine shows a well-characterized anti-inflammatory effect [55, 56], our data showed that adenosine did not prevent TNF- α and IL-1 β release in LPS-stimulated astrocytes, in accordance with Kucher and Neary [57]. Moreover, caffeine did not antagonize the anti-inflammatory effect of guanosine, indicating that its effect do not occur via an adenosine receptor-dependent mechanism.

In a previous report, D’Alimonte investigated the protective effects of guanosine in inflammatory processes and showed that in mouse microglial cells, guanosine treatment inhibited TNF- α release [58]. Additionally, consistent with our findings, Jackson and Mi showed that guanosine inhibited the LPS-induced production of TNF- α and IL-1 β in an in vivo experimental model [46]. Our group recently demonstrated in the C6 astroglial cell line that the HO-1 pathway was involved in the anti-inflammatory activity of guanosine under azide exposure and that its glioprotective effects were associated with decreased neuroinflammation and oxidative stress [30]. HO-1 and its degradation product, carbon monoxide, can suppress the expression of pro-inflammatory mediators and NFκB translocation, preventing triggering of the inflammatory cascade.

Astrocytes are activated after a systemic or central injury, which progresses into a neuroinflammatory response, and initially, they exhibit neuroprotective effects to maintain CNS

Fig 3 Effects of guanosine on oxidative stress parameters. Cells were incubated in serum-free DMEM/F12 with 100 μ M guanosine (GUO) for 1 h, followed by the addition of 10 μ g/mL LPS for 3 h. The cells were co-incubated with 10 μ M ZnPP IX along with the guanosine. **a** Mitochondrial membrane potential ($\Delta\Psi_m$), **b** ROS production, **c** superoxide levels, and **d** nitrite levels were measured as described in the “Materials and methods” section. Data represent the mean $\pm$ S.E.M. of four independent experiments that were performed in triplicate. Differences between groups were statistically analyzed using two-way ANOVA, followed by Tukey’s test. Values of $P < 0.05$ were considered significant. *a* indicates differences from control conditions, and *b* differences from LPS

homeostasis. However, their overactivation leads to both protective and destructive effects: These effects primarily depend on the signaling molecules that they secrete and respond to. Activated astrocytes release TNF- α , and TNF- α is the first signal that enhances other pro-inflammatory cytokines such

Fig 4 Guanosine prevents the LPS-mediated decrease in GSH levels. Cells were incubated in serum-free DMEM/F12 with 100 μ M guanosine (GUO) for 1 h, followed by the addition of 10 μ g/mL LPS for 3 h. The cells were co-incubated with 10 μ M ZnPP IX along with the guanosine. GSH levels were measured as described in the “Materials and methods” section. Data represent the mean $\pm$ S.E.M. of three independent experiments that were performed in triplicate. Differences between groups were statistically analyzed using two-way ANOVA, followed by Tukey’s test. Values of $P < 0.05$ were considered significant. *a* indicates differences from control conditions, and *b* differences from LPS

as IL-1 β , which has been shown to play a role in apoptosis and blood–brain barrier disruption [59]. Moreover, TNF- α can potentiate glutamatergic excitotoxicity by inhibiting glutamate transport in astrocytes, and by increasing the localization of ionotropic glutamate receptors to synapses [60]. Here, we provide the first demonstration that guanosine inhibits TNF- α and IL-1 β production in hippocampal primary astrocyte cultures and that co-incubation with HO-1 inhibitor blocked these positive effects of guanosine. Avoiding the release of these mediators is crucial for the management of inflammatory diseases because both these cytokines are essential for the production of other cytokines, which exacerbate inflammatory processes in the brain [61].

TNF- α is a clear activator of the signal transduction pathway of NF κ B. It has been suggested that NF κ B plays an important role in sustaining the vicious cycle of inflammatory response and in maintaining interactions between astrocytes and neurons [62]. Numerous studies have shown that suppressing pro-inflammatory astroglial NF κ B signaling could benefit clinical outcomes in cases of neuroinflammatory-related diseases [47]. The activation of this transcription factor promotes NO production due to growth in the expression of iNOS, which could lead to oxidative stress and neuronal death [35]. In line with this, we showed that guanosine, through HO-1 activation, prevented NF κ B translocation and consequently inhibited NO

production. This is in accordance with a previous report from our group that demonstrated that guanosine modulates iNOS and NO expression in C6 astroglial cells [30].

NO also mediates disruption of the outer mitochondrial membrane, which contributes to cell death by bioenergetic failure, the loss of intramitochondrial contents, an increase of ROS production, and the release of signaling molecules that regulate cellular apoptosis [63]. Under LPS exposure, mitochondrial metabolic status ($\Delta\Psi_m$) was affected, indicating impairment of mitochondrial function in the hippocampal astrocytes. Mitochondrial protection in astrocytes, which came to the fore after treatment with guanosine, plays a fundamental role in maintaining the energetic balance of the brain and in producing the antioxidants that contribute to neuronal protection. ROS and superoxide production is widely attributed to mitochondria and NADPH oxidase complexes [64, 65]. Reinforcing the previously reported antioxidant effect, we demonstrated here that guanosine reduced LPS-induced oxidative parameters. It is possible that guanosine may exert its effects by direct radical scavenging activity and may also modulate signaling pathways, such as the HO-1 pathway, that control antioxidant defenses.

The antioxidative effect of guanosine was also demonstrated by its modulation of the homeostasis of GSH, the major nonenzymatic antioxidant in the CNS [66]. Our results showed that guanosine was able to prevent the LPS-induced decrease in GSH content that occurred through HO-1. This pathway is regulated by Nrf-2, a neuroprotective transcription factor that modulates several detoxification genes that encode antioxidant proteins, such as the GSH system [35]. An increase in GSH levels in glial cells confers protection against neurodegenerative disorders, such as Alzheimer's and Parkinson's disease [67]. On the other hand, the depletion of GSH content in astrocytes induces inflammatory response, cytotoxicity, and impairment of glutamate transporter activity [68, 69].

To the best of our knowledge, the present study provides the first demonstration that guanosine can inhibit the inflammatory response induced by LPS in primary cultures of hippocampal astrocytes. The protective mechanism of guanosine involves a decrease in pro-inflammatory and oxidative mediators as well as an increase in GSH content and defense against mitochondrial dysfunction. All of its effects were mediated by the HO-1 pathway and together may contribute to the maintenance of brain homeostasis. Given the glioprotective actions that have already been described for guanosine, its modulatory effects through HO-1 could help explain the mechanisms by which guanosine protects astrocytes against different stimuli and highlight the potential role of guanosine against neuroinflammatory-related diseases.

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Conflict of interest The authors declare that there are no conflicts of interest.

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