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ORIGINAL PAPER

Protective Effects of Resveratrol on Hydrogen Peroxide Induced Toxicity in Primary Cortical Astrocyte Cultures

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Abstract It is well established that the brain is particularly susceptible to oxidative damage due to its high consumption of oxygen and that astrocytes are involved in a variety of important activities for the nervous system, including a protective role against damage induced by reactive oxygen species (ROS). The use of antioxidant compounds, such as polyphenol resveratrol found in red wine, to improve endogenous antioxidant defenses has been proposed for neural protection. The aim of this study is to evaluate the putative protective effect of resveratrol against acute H_2O_2-induced oxidative stress in astrocyte cultures, evaluating ROS production, glutamate uptake activity, glutathione content and S100B secretion. Our results confirm the ability of resveratrol to counteract oxidative damage caused by H_2O_2, not only by its antioxidant properties, but also through the modulation of important glial functions, particularly improving glutamate uptake activity, increasing glutathione content and stimulating S100B secretion, which all contribute to the functional recovery after brain injury.

Keywords Astrocyte · Glutathione · Glutamate uptake · Oxidative stress · S100B protein · Resveratrol

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Introduction

Reactive oxygen species (ROS) are products of general metabolism and play important roles in several physiological cellular functions. Imbalances in the generation of ROS and cellular antioxidant defenses lead to oxidative stress, which results in oxidative damage (see [1] for a review). ROS include hydrogen peroxide (H_2O_2) that is normally produced in the tissues through reactions catalyzed by superoxide dismutase (SOD) and oxidases. H_2O_2 is removed predominantly by the antioxidant enzymes catalase and glutathione peroxidase [2], however, H_2O_2 may also act as a regulator of signal pathways since it has the ability to cross the plasma membrane and increase cytosolic calcium [3, 4].

Oxidative stress has long been associated with the development of pathological conditions in brain tissue such as ischemia, inflammation and degenerative diseases including Alzheimer's disease, Huntington and Parkinsons's disease [5, 6]. It is well established that the brain is particularly susceptible to oxidative damage due to its high consumption of oxygen and high quantities of polyunsaturated fatty acids. In brain, SOD and monoaminoxidases A and B (involved in the catecholamine and serotonin catabolism) are the main sources of H_2O_2.

Astrocytes are the most abundant cell type in the brain and involved in a variety of important activities for the nervous system, including a protective role against damage induced by ROS [7]. Glutathione is a major antioxidant of the brain [8] present in higher amounts in astrocytes [9]. In fact, astrocytes are able to uptake cystine, convert cystine to cystein and incorporate cystein in glutathione [10]. Neurons depend on the astrocyte content of cystein and glutathione to synthesize their own glutathione, demonstrating the importance of the interaction between astrocyte

and neurons [11]. Moreover, there is much evidence to demonstrate another crucial role of astrocytes in glutamate metabolism. The impairment of glutamate transporters causes excitotoxicity and leads to increased ROS production and consequent cell damage [12]. In addition, ROS directly impair glutamate transporters in astrocytes and neurons [13].

S100B is a calcium-binding protein that is primarily expressed and secreted in the central nervous system by astroglia. This protein, at nanomolar concentrations, stimulates neuronal survival in vitro and is able to protect hippocampal neurons against glutamate toxicity [14]. Although the mechanism of S100B secretion is unknown, it appears to be related to glutamate uptake activity [15] and is affected by oxidative stress [16].

Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) is a polyphenol found in grapes and red wine with diverse established biological activities, such as antioxidant, anti-inflammatory, cardioprotective and anticarcinogenic roles [17]. Recently, a number of studies have focused on the neuroprotective effects of resveratrol, demonstrating that this compound attenuates β-amyloid toxicity [18] and protects against cerebral ischemic injury [19] and kainic acid-induced excitotoxicity [20]. However, little is known about the effect of resveratrol on astrocytes. The aim of this study is to evaluate the putative protective effect of resveratrol against acute H_2O_2-induced oxidative stress in astrocyte cultures, evaluating ROS production, glutamate uptake activity, glutathione content and S100B secretion.

Experimental procedure

Material

Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene, approximately 99% purity), poly-D-lysine, γ-glutamylhydroxamate and anti-S100B antibody (SH-B1) were purchased from Sigma. L-[2,3-^{3}H]glutamate was purchased from Amersham (specific activity 33 Ci/mmol). Fetal calf serum was purchased from Cultilab (São Paulo, Brazil). Dulbecco's modified Eagle's medium (DMEM) and other materials for cell culture were purchased from Gibco. DCF was provided from Molecular probe. H_2O_2 was obtained from Merck.

Astrocyte cultures

Primary cortical astrocyte cultures were prepared as previously described [21]. Briefly, cerebral cortices of newborn Wistar rats (1–2-days-old) were removed, placed in Ca^{2+} and Mg^{2+}-free buffer saline solution, pH 7.4, containing (in mM): 137 NaCl; 5.36 KCl; 0.27 Na_2HPO_4; 1.1 KH_2PO_4 and 6.1 glucose. The cortices were cleaned of meninges and mechanically dissociated. After centrifugation at 1,000 rpm for 5 min the pellet was resuspended in DMEM (pH 7.6) supplemented with 8.39 mM HEPES; 23.8 mM $NaHCO_3$; 0.1% fungizone; 0.032% garamycin and 10% fetal calf serum (FCS). The cells were plated at a density of 2×10^5 cells per cm^2 onto 24 well-plates pretreated with poly-L-lysine. Cultures were maintained in 5% CO_2/95% air at 37 °C and allowed to grow to confluence and used at 15–20 days in vitro.

Immunocytochemistry for GFAP

Cells in basal conditions were fixed for 20 min with 4% paraformaldehyde in phosphate buffer (PBS): 2.9 mM KH_2PO_4, 38 mM $Na_2HPO_4 7H_2O$, 130 mM NaCl, 1.2 mM KCl, rinsed with PBS and permeabilized for 10 min in PBS containing 0.2% Triton X-100. Fixed cells were then blocked for 60 min with PBS containing 0.5% bovine serum albumin and incubated overnight with polyclonal anti-GFAP (Dako, 1:200) followed by peroxidase-conjugated anti-rabbit IgG for 2 h. Finally, the cells were treated with 0.05% diaminobenzidine (Sigma) containing 0.01% hydrogen peroxide for 10 min [22]. Cells were viewed with a Nikon inverted microscope and images transferred to a computer with a digital camera (Sound Vision Inc., USA).

H_2O_2 treatment

Prior to H_2O_2 insult, cell cultures were pre-incubated in serum-free DMEM for 30 min, containing or not resveratrol at a concentration of 50 μM. Media were then replaced and cells were incubated with serum-free DMEM containing, or not, freshly made 100 μM H_2O_2 for another 30 min. Cells were then maintained in serum-free DMEM for 24 h and possible resulting changes were evaluated at 1 h and 24 h after H_2O_2 exposure. Importantly, cells pre-incubated with resveratrol were supplemented with this compound during all replacements, i.e., during H_2O_2 insult and afterwards. In all analyzed parameters, the results obtained with vehicle (0.25% ethanol) were not different from those obtained in basal conditions without ethanol.

Evaluation of intracellular ROS production

Intracellular ROS production was detected using the non-fluorescent cell permeating compound, 2′–7′-dichlorofluorescein diacetate (DCF-DA). DCF-DA is hydrolyzed by intracellular esterases and then oxidized by ROS to a fluorescent compound 2′–7′-dichlorofluorescein (DCF). Astrocytes were treated with DCF-DA (10 μM) for 30 min at 37°C and rinsed with DMEM without serum. Cells were viewed with a Nikon inverted microscope using a TE-FM

Epi-Fluorescence accessory and images transferred to a computer with a digital camera (Sound Vision Inc, USA). All images are representative fields from at least three experiments carried out in triplicate. In another set of experiments, following DCF-DA exposure, the cells were rinsed and then scraped into PBS 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.

Glutamate uptake assay in astrocytes

Glutamate uptake was performed as previously described [21]. Briefly, cortical astrocytes were incubated at 37°C in a Hank's balanced salt solution (HBSS, pH 7.4) containing: 135 mM NaCl; 3.1 mM KCl; 1.2 mM $CaCl_2$; 1.2 mM $MgSO_4$; 0.5 mM KH_2PO_4; 2 mM glucose, 0.1 mM L-glutamate and 0.33 μCi/ml L-[2,3-^{3}H]glutamate for 7 min. Na^+-free medium was prepared by replacing NaCl with choline chloride. Incubation was terminated by removal of the medium and rinsing the cells twice with ice-cold HBSS. Cells were then resuspended in a lysis solution containing 0.1 N NaOH and 0.01% SDS. Radioactivity was measured with a scintillation counter.

Total glutathione assay

Total glutathione content was determined by a slightly modified assay, as described previously [23, 24]. Briefly, cells were scraped in phosphate buffered saline (0.01 M, pH 7.6), 6.3 mM EDTA (pH 7.5) and Triton-X (0.05%) and protein was precipitated with 1% sulfosalicylic acid. Supernatant was assayed with 462.6 μM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), 0.5 U/ml glutathione reductase, and 0.3 mM NADPH; reduced DTNB was measured at 412 nm.

Immunocontent of S100B

The S100B concentration was determined in the culture medium at 1 h and 24 h. ELISA for S100B was carried out as described previously with modifications [25]. Briefly, 50 μl of sample plus 50 μl of Tris buffer were incubated for 2 h on a microtiter plate previously coated with monoclonal anti-S100B (SH-B1, from Sigma). Polyclonal anti-S100 (from DAKO) was incubated for 30 min and peroxidase-conjugated anti-rabbit antibody was then added for a further 30 min. The color reaction with *o*-phenylenediamine was measured at 492 nm.

Other measurements

Protein content was measured by Lowry's method using BSA as a standard [26]. Extracellular S100B content was referred to as "secretion," based on cell integrity measurement (data not shown) with LDH activity by a colorimetric commercial kit (from Doles, Brazil) and Trypan blue exclusion assay [27].

Statistical analysis

Data from at least three independent experiments are presented as means ± S.E.M and were analyzed statistically by one-way analysis of variance (ANOVA) followed by the Tukey's test. Values of $P < 0.05$ were considered to be significant. All analyses were carried out in an IBM compatible PC using the Statistical Package for Social Sciences (SPSS) software.

Results

Resveratrol inhibited intracellular ROS production induced by H_2O_2

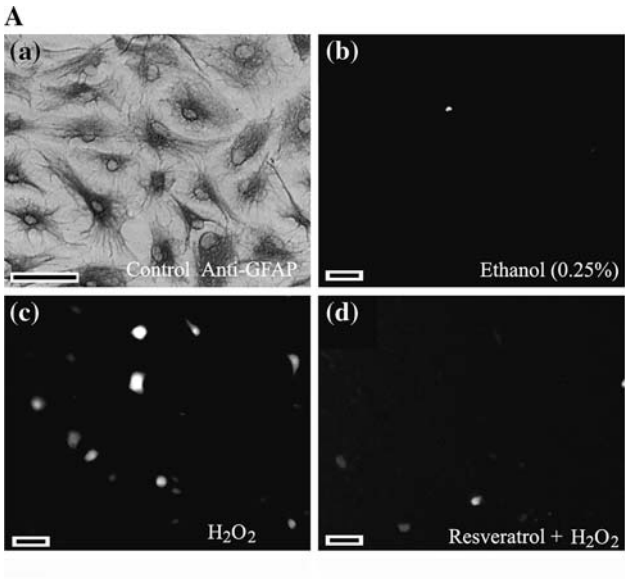
Immunocytochemistry for GFAP showed typical polygonal flat morphology and astrocyte purity >95% (Fig. 1A, panel a). In order to investigate the effect of resveratrol on astrocytes exposed to 100 μM H_2O_2, we previously characterized the changes in intracellular ROS production using DCF. One hour after the insult with H_2O_2 we observed an increase in ROS production, as expected (Fig. 1A, panel c), whilst addition of 50 μM resveratrol prevented this alteration (Fig. 1A, panel d). These observations at 1 h after H_2O_2 exposure were confirmed by a quantification of DCF using a microplate reader (Fig. 1B). Twenty-four hours afterwards, no alteration was observed (data not shown).

Effect of resveratrol on glutathione content after H_2O_2 insult

H_2O_2 (at 100 μM) induced a decrease in glutathione levels after 1 h in astrocytes (Fig. 2A); conversely, resveratrol increased glutathione content. Moreover, resveratrol (at 50 μM) not only prevented the H_2O_2 effect, but increased glutathione at 1 h after H_2O_2. The H_2O_2-induced decrease in glutathione was not observed at 24 h; however, the resveratrol-induced glutathione increment was maintained at this time (Fig. 2B).

Effect of resveratrol on glutamate uptake after H_2O_2 insult

We observed that 100 μM H_2O_2 decreased glutamate uptake at 1 h after insult. Resveratrol (at 50 μM), added together with H_2O_2, was able not only to prevent this decrease, but to induce an increment in glutamate uptake



B

Ethanol (0.25%)	H_2O_2	Resveratrol + H_2O_2
106.0 ± 8.5	151.6 ± 4.8***	54.8 ± 6.7**

Fig. 1 Effect of resveratrol on intracellular ROS accumulation 1 h after H_2O_2 induced damage in astrocyte cultures. In **A**, cells were transferred to serum-free DMEM containing, or not, resveratrol (50 μM) for 30 min and then exposed or not to H_2O_2 (100 μM) for another 30 min. The level of intracellular ROS was measured with DCF-DA. Panel a depicts immunocytochemistry for GFAP under basal conditions. Representative fluorescent microscope images of primary astrocyte cultures in basal conditions (panel b) or exposed to H_2O_2 (panel c) or exposed to H_2O_2 in presence of resveratrol (panel d). All images are representative fields of at least three independent experiments carried out in triplicate. Scale bar = 50 μm. In **B**, the fluorescence was measured in a fluorescence microplate reader (excitation 485 nm and emission 520 nm). The basal condition without ethanol was assumed as being 100%. Statistically significant differences from basal, by one-way ANOVA followed by Tukey's multiple variation test, are indicated: ** $P < 0.01$, *** $P < 0.001$

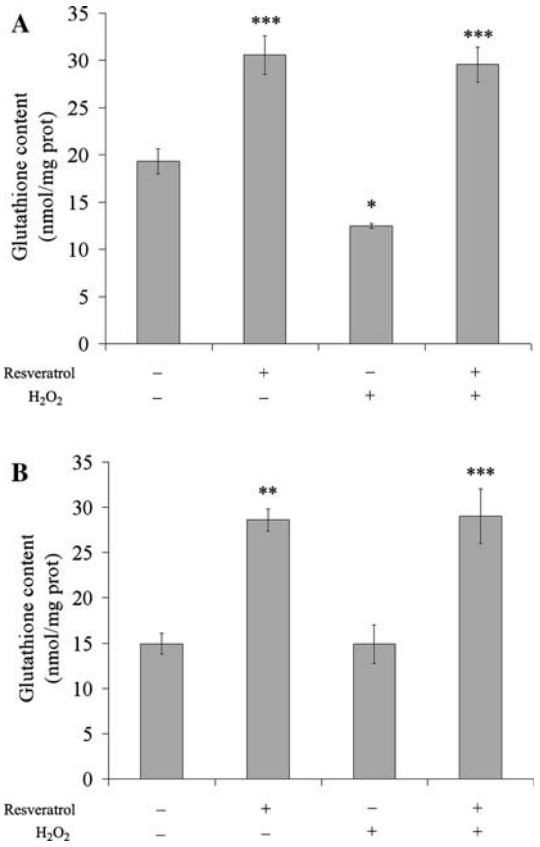


Fig. 2 Glutathione content in primary astrocyte cultures treated with resveratrol. Cells were transferred to serum-free DMEM containing or not resveratrol (50 μM) for 30 min and then exposed or not to H_2O_2 (100 μM) for another 30 min. Glutathione content was measured by a colorimetric assay with DTNB 1 (in **A**) and 24 h (in **B**) after H_2O_2 exposure. The data represent the mean ± SEM values of 4 independent experiments performed in triplicate. Statistically significant differences from controls, as determined by one-way ANOVA followed by Tukey's multiple variation test, are indicated: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

(Fig. 3A). Resveratrol per se did not increase glutamate uptake activity. Glutamate uptake activity was recovered 24 h after H_2O_2 insult (Fig. 3B). Interestingly, the combined exposure of resveratrol and H_2O_2 resulted in a persistent increase in glutamate uptake at 24 h after insult.

Effect of resveratrol on S100B secretion after H_2O_2 insult

About 100 μM H_2O_2 exposure caused an increase in S100B secretion at 1 h and 50 μM resveratrol, *per se*, was not able to change basal secretion. H_2O_2 insult in the presence of resveratrol, however, resulted in a decrease in S100B (Fig. 4A). Measuring extracellular levels of S100B, we found a decrease 24 h after H_2O_2 exposure. Conversely, resveratrol addition resulted in an increment of extracellular S100B at 24 h, even after H_2O_2 exposure (Fig. 4B).

Discussion

During the last decade, several studies have demonstrated resveratrol to be a potent antioxidant that protects against different ROS [17]. Furthermore, other important proprieties of resveratrol have been also reported, such as anti-inflammatory properties, modulation of diverse signaling pathways and anti-carcinogenic effects [17]. In the brain, resveratrol shows promise as a compound that may be useful in neurodegenerative processes and acute situations of injury, where the astrocytes act as potential therapeutic targets [28, 29].

Many studies in non-neural cell preparations have shown that increased ROS, induced by a brief H_2O_2 insult, was prevented in the presence of resveratrol (e.g., [30, 31]). In addition, resveratrol attenuates intracellular ROS accumulation and prevents H_2O_2-induced apoptosis in PC12

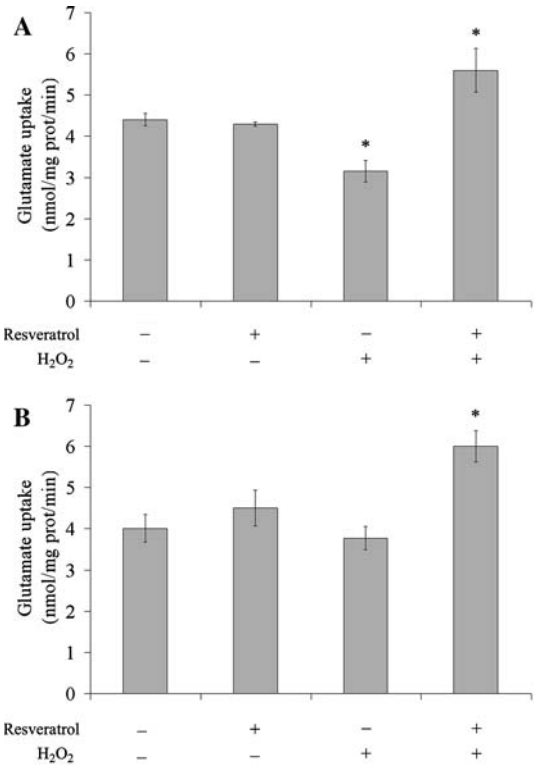


Fig. 3 Effect of resveratrol on glutamate uptake in astrocyte culture 1 h (**A**) and 24 h (**B**) after the insult with H_2O_2. Cells were transferred to serum-free DMEM containing or not resveratrol (50 μM) for 30 min and then exposed or not to H_2O_2 (100 μM) for another 30 min. After 1 (panel A) or 24 h (panel B) cell culture media were replaced with HBSS and incubated with [^{3}H]-glutamate for 7 min. The data represent the mean ± SEM values from 4 to 5 independent experiments performed in triplicate. Statistically significant differences from controls, as determined by one-way ANOVA followed by Tukey's multiple variation test, are indicated: * $P < 0.05$

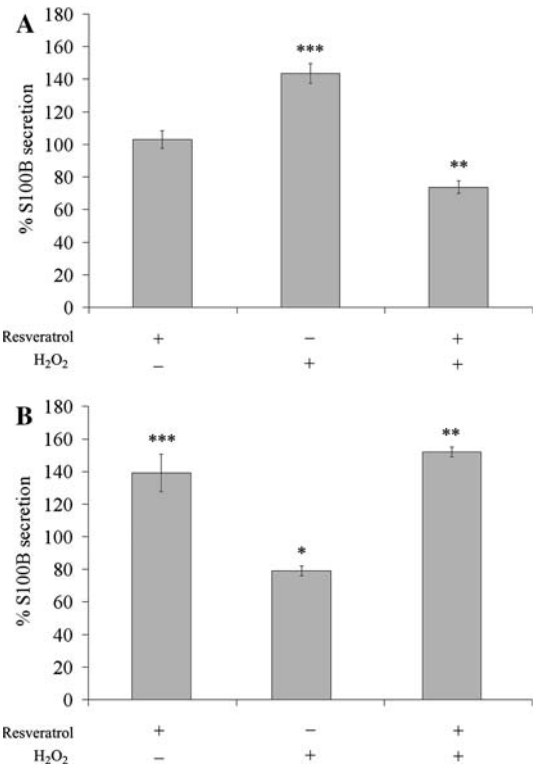


Fig. 4 Effect of resveratrol on S100B secretion in astrocyte culture after the insult with H_2O_2. Cells were transferred to serum-free DMEM containing or not resveratrol (50 μM) for 30 min and then exposed or not to H_2O_2 (100 μM) for another 30 min. Extracellular S100B was measured by ELISA 1 h (in panel **A**) and 24 h (in panel **B**) after insult with H_2O_2. Control S100B secretion presented mean values of 0.41 and 1.92 ng/ml at 1 h and 24 h, respectively; these values were assumed as 100%. Each value is the percentage mean ± SEM of 4–5 independent experiments performed in triplicate. Different letters indicate statistical difference of extracellular S100B levels from the control, determined by one-way ANOVA followed by Tukey's multiple variation test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

cells [32]. In agreement with these studies, we observed a decrease in DCF oxidation when primary astrocyte cultures were treated with resveratrol. It is important to emphasize that H_2O_2 exposure occurred for exactly 30 min and that medium was then replaced before DCF assay and other measurements. Astrocytes, the most abundant glial cells, have an important protective role against ROS in CNS. Astrocytes can rapidly remove H_2O_2 and release antioxidant and trophic substances, protecting neurons against oxidative stress [33]. Moreover, astrocytes are involved in numerous other functions including synthesis and secretion of neurotrophic substances, uptake of neurotransmitters, and are essential for metabolic energy support of neurons [34]. It is important to mention that we used ethanol as a vehicle (at 0.25%) for resveratrol and no apparent oxidative stress was caused by ethanol per se in primary astrocyte culture, confirming the ethanol resistance of these cells, which could even protect neurons against ethanol-induced damage [35].

Several brain disorders are accompanied by a decrease in glutathione and other indications of oxidative stress [36]. Resveratrol was able to induce a fast and persistent increment of glutathione in primary astrocyte cultures. As expected, H_2O_2 induced a decrease in total glutathione content, which was prevented in cells that were pre-incubated and incubated in the presence of resveratrol. Glutathione constitutes a non-enzymatic scavenger and substrate for glutathione peroxidases, furthermore, astrocytes export glutathione precursors for neuronal synthesis [37]. Our data indicate an important regulation of glutathione content by resveratrol, and the provision of protection against H_2O_2 injury.

Astrocytes are key elements that regulate glutamate levels in the synaptic cleft. It has been well characterized that glutamate transporters are very sensitive to oxidative stress and this vulnerability is commonly associated to

neurodegenerative diseases and some acute brain injuries [13]. Previous studies have reported that H_2O_2 significantly affects glutamate transporter activity in cultured rat cortical astrocytes [e.g., 38]. Resveratrol at 50 μM was able to improve glutamate uptake activity during H_2O_2 insult, but was unable to alter basal glutamate uptake. Our results corroborate the idea that resveratrol provides protection in the presence of oxidative damage induced by H_2O_2. Recently, resveratrol was demonstrated to increase basal glutamate uptake in C6 glioma cells [16]. Reasons for this difference are unclear at this moment, but could be due to differences in glutamate transporters [39] and/or the redox environment of these cultures. Regardless of this aspect, our results indicate the beneficial effect of resveratrol on glutamate uptake activity. This effect cannot be attributed exclusively to the antioxidant property of resveratrol, since it persists 24 h afterwards. The long-term effect of this compound could involve changes in glutamate activity, mediated by protein kinases [18, 40].

Another possible effect that may be afforded by resveratrol neuroprotection is its ability to induce S100B secretion. Many clinical studies have suggested the elevation of peripheral S100B as a marker of brain damage [41, 42]. However, based on the neuroprotection observed in neural cultures of this protein (see [43] for a review) and on its transitory extracellular increment in acute brain injury it has been proposed that, far from being a negative determinant of outcome, S100B may improve functional recovery following acute brain injury [42].

It should be noted that S100B secretion was transiently increased (first hour) after H_2O_2 insult. Interestingly, H_2O_2-induced S100B secretion showed a contrasting profile in our experiment, dependent on the presence of resveratrol. When oxidative damage was induced in astrocytes treated with H_2O_2, an increase in S100B secretion was detected one hour after; conversely, when astrocytes were pre-incubated with resveratrol a decrease was observed in S100B secretion. Resveratrol did not change S100B secretion during the first hour of insult, but induced a late increment (24 h afterwards) of extracellular levels of S100B. In C6 glioma, a late increment (48 h afterwards) in the S100B secretion also was induced by resveratrol [16].

Recently, we proposed that an increment of glutamate influx (as observed in excitotoxic conditions) could result in a decrease in S100B secretion [15]. In agreement, H_2O_2-induced damage could explain the opposite variations in S100B secretion and glutamate uptake, observed under our experimental conditions during the first hour. However, the mechanism by which pre-incubation with resveratrol decreases S100B secretion, stimulated by H_2O_2, during the first hour is not clear at the moment. The limited knowledge about the mechanism of S100B secretion, as well as the molecular signaling involved contributes to maintain this doubt. However, it may be postulated that resveratrol stimulates S100B secretion, during the long-term, which, in turn, stimulates neuronal survival and activity during brain injury and recovery.

Resveratrol has also been shown to be beneficial against a number of brain disorders, including spinal cord and cerebral ischemia, Parkinson disease, amyotrophic lateral sclerosis, diabetes mellitus, epilepsy, brain tumors and aging [19, 44, 45]. In the same vein, our results sustain and corroborate the benefits of resveratrol in CNS. Moreover, this study complements previous results with C6 glioma cells. Some limitations of this study, however, should be considered; firstly, further studies involving co-culture with neurons are necessary to confirm the protection of neurons mediated by astrocytes; secondly, the concentration of resveratrol used in this study is apparently elevated when compared to its concentration in tissues (less than 2 μM). In this investigation, based on recent studies, we used 50 μM [16, 46]. Some authors have proposed that it would be reasonable to conceive that resveratrol at levels ≤25 μM could be potentially useful in experimental conditions [45]. It is important to mention that we found that 25 μM resveratrol also induced a similar increment in glutathione content in primary astrocyte cultures treated for 24 h [28]. Finally, a limitation to be considered is the exogenous addition of H_2O_2 as a model of oxidative stress in cell culture; however, the quantity of exogenous or generated H_2O_2 appropriate for mimicking the pathological and/or physiological conditions in which this compound is involved remains questionable in the several models utilized to study oxidative stress [47].

In conclusion, the results presented here attest the ability of resveratrol to counteract oxidative damage caused by H_2O_2, not only via its antioxidant properties, but also through the modulation of important astrocytic functions, particularly improving glutamate uptake activity, increasing glutathione content and stimulating S100B secretion. All these changes favor the neural functional recovery after brain injury.

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