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ORIGINAL PAPER

Resveratrol Increases Glutamate Uptake, Glutathione Content, and S100B Secretion in Cortical Astrocyte Cultures

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Abstract Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) is a polyphenol present in grapes and red wine, which has antioxidant properties and a wide range of other biological effects. In this study, we investigated the effect of resveratrol, in a concentration range of 10–250 μM, on primary cortical astrocytes; evaluating cell morphology, parameters of glutamate metabolism such as glutamate uptake, glutamine synthetase activity and glutathione total content, and S100B secretion. Astrocyte cultures were prepared of cerebral cortex from neonate Wistar rats. Morphology was evaluated by phase-contrast microscopy and immunocytochemistry for glial fibrillary acidic protein (GFAP). Glutamate uptake was measured using L-[2,3-^{3}H]glutamate. Glutamine synthetase and content of glutathione were measured by enzymatic colorimetric assays. S100B content was determined by ELISA. Typical polygonal morphology becomes stellated when astrocyte cultures were exposed to 250 μM resveratrol for 24 h. At concentration of 25 μM, resveratrol was able to increase glutamate uptake and glutathione content. Conversely, at 250 μM, resveratrol decreased glutamate uptake. Unexpectedly, resveratrol at this high concentration increased glutamine synthetase activity. Extracellular S100B increased from 50 μM upwards. Our findings reinforce the protective role of this compound in some brain disorders, particularly those involving glutamate toxicity. However, the underlying mechanisms of these changes are not clear at the moment and it is necessary caution with its administration because elevated levels of this compound could contribute to aggravate these conditions.

Keywords Astrocyte · GFAP · Glutamate uptake · Glutathione · Resveratrol · S100B

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Introduction

Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene), a polyphenol present in grapes and red wine, has antioxidant and anti-inflammatory proprieties and a wide range of other biological effects. Many studies now attest to the cardioprotective and chemopreventive effects of this compound (Frémont 2000; Baur and Sinclair 2006; Pervaiz 2004). A neuroprotective action of resveratrol has been proposed in many conditions of brain injury (Dore 2005), including

damage mediated by glutamate receptors (Wang et al. 2004; Gao et al. 2006), but the underlying mechanism is not fully understood.

Glutamate is the major excitatory neurotransmitter in the central nervous system and its accumulation is implicated in neurodegenerative disorders. Astrocytes, the main glial cells of the brain, are responsible for major glutamate transport, regulating its extracellular levels through glutamate sodium-dependent transporters (GLAST and GLT-1) (Hertz 2006). Glutamate accumulation in the extracellular space is known to be toxic not only to neurons but also to astrocytes (Matute et al. 2002; Chen et al. 2000). Moreover, astrocytes have a specific enzyme glutamine synthetase (GS) that catalyses the amidation reaction of glutamate to form glutamine, which is exported to neurons, allowing the synthesis of not only glutamate, but also GABA by the glutamate–glutamine cycle (Bak et al. 2006). We recently showed that resveratrol increases glutamate uptake and GS activity in C6 glioma cells (Dos Santos et al. 2006). Moreover, astrocytic glutamate uptake is also essential for maintaining glutathione levels, the main antioxidant of the brain (Dringen 2000). Indeed, neurons depend on astrocytes for precursors to synthesize their own glutathione (Dringen et al. 1999). Brain tissue is particularly vulnerable to oxidative damage, due to its high consumption of oxygen and the consequent generation of high quantities of reactive oxygen species during oxidative phosphorylation (Castagne et al. 1999). Thus, astrocyte antioxidant defense is a key element to physiological brain activity.

Astrocytes are also involved in many other neural functions, such as maintenance of ion homeostasis, energetic metabolism, and secretion of neurotrophic factors. Among these trophic factors is the S100B protein, a calcium-binding protein that is secreted from astrocytes in a glutamate transporter-dependent manner (Tramontina et al. 2006). This protein has neurotrophic activity at nanomolar levels in the extracellular medium (Donato 2001; Van Eldik and Wainwright 2003).

Here, we investigate the potential neuroprotective effects of resveratrol on primary cortical astrocytes, evaluating cell morphology, parameters of glutamate metabolism such as glutamate uptake, glutamine synthetase activity and glutathione total content, and S100B secretion.

Methods

Materials

Resveratrol (approximately of 99% purity), poly-D-lysine, γ-glutamylhydroxamate, and antibody anti-S100B (SH-B1) were purchased from Sigma and polyclonal anti-GFAP from DAKO. L-[2,3-^{3}H]Glutamate was purchased from Amersham (specific activity 33 Ci/mmol). Fetal calf serum (FCS) was purchased from Cultilab (São Paulo, Brazil). Dulbecco's modified Eagle's medium (DMEM) and other materials for cell culture were purchased from Gibco.

Astrocyte cultures

Primary cortical astrocyte cultures were prepared as previously described (Gottfried et al. 1999). Briefly, cerebral cortex of newborn Wistar rats (1- to 2-day-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% FCS. The cells were plated at a density of 2×10^5 cells per cm^2 onto 24-well plates pre-treated 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.

Drug treatment

Astrocytes were incubated for 24 h in DMEM (pH 7.4) without serum in the absence or presence of resveratrol at concentrations of 10, 25, 50, 100, and 250 μM. In all analyzed parameters, the results obtained with vehicle (0.25% ethanol) were not different from those obtained in basal conditions without ethanol.

Immunocytochemistry and cell morphology

After 24 h, cells treated with resveratrol were fixed for 20 min with 4% paraformaldehyde in phosphate

buffer (PBS): 2.9 mM KH_2PO_4, 38 mM Na_2H-$PO_4{\cdot}7H_2O$, 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) for 2 h. Finally, the cells were treated with 0.05% diaminobenzidine (Sigma) containing 0.01% hydrogen peroxide for 10 min (Gottfried et al. 2003). Cells were viewed with a Nikon inverted microscope and images transferred to a computer with a digital camera (Sound Vision Inc., Wayland, MA, USA). All images are representative fields from at least three experiments carried out in triplicate.

Glutamate uptake assay in astrocytes

Glutamate uptake was performed as previously described (Gottfried et al. 2002). 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.

Glutamine synthetase activity

The enzymatic assay was performed according to Petito et al. (1992) with some modifications. Briefly, homogenate (0.1 ml) was added to 0.1 ml of reaction mixture containing (in mM): 10 $MgCl_2$; 50 L-glutamate; 100 imidazole–HCl buffer (pH 7.4); 10 2-mercaptoethanol; 50 hydroxylamine–HCl; 10 ATP and incubated for 15 min at 37°C. The reaction was stopped by the addition of 0.4 ml of a solution containing (in mM): 370 ferric chloride; 670 HCl; and 200 trichloroacetic acid. After centrifugation, the supernatant was measured at 530 nm and compared to the absorbance generated by standard quantities of γ-glutamylhydroxamate treated with ferric chloride reagent.

Total glutathione assay

Total glutathione content was determined by a slightly modified assay, as described previously (Allen et al. 2001; Tietze 1969). Briefly, cells were scraped in phosphate-buffered saline (0.01 M, pH 7.6), 6.3 mmol edetic acid (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, 6, and 24 h. Cells were washed and scraped at 24 h for measurement of intracellular S100B content. ELISA for S100B was carried out as described previously with modifications (Tramontina et al. 2000). 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.

Cell integrity

Total intracellular lactate dehydrogenase (LDH) activity after resveratrol exposure was determined in lysed cells with 0.2% Triton X-100 by a colorimetric assay kit (from Doles, Goiânia, Brazil).

Protein content

The total protein content was determined by the method of Lowry et al. (1951), using bovine serum albumin as standard.

Statistical analysis

Data from at least three independent experiments are presented as mean ± 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

Cell integrity and morphology in the presence of resveratrol

Phase-contrast images and immunocytochemistry for GFAP show typical polygonal astrocyte morphology (Fig. 1, panels **a** and **b**, respectively). Exposure to 25 μM resveratrol (panels **c** and **d**) had no effect 24 h afterwards, but process-bearing cells (stellation) were observed following 250 μM resveratrol exposure (panels **e** and **f**). Membrane integrity, based on LDH activity assay, was preserved after resveratrol (from 10 to 250 μM) or vehicle per se (0.25% ethanol) exposure (data not shown).

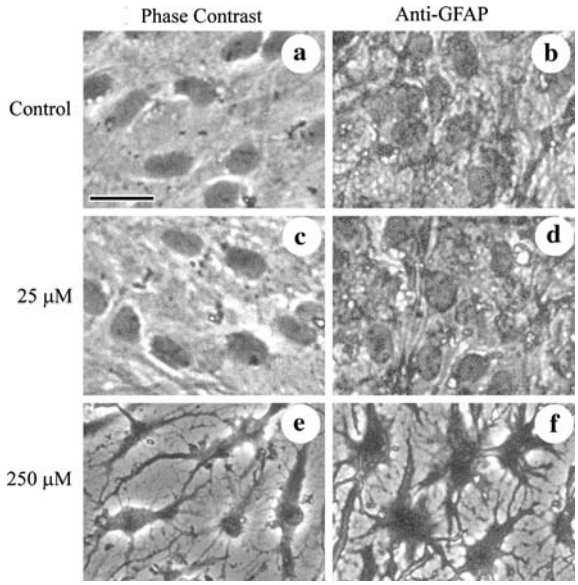

Fig. 1 Phase-contrast microscopy and immunocytochemistry showing morphological changes in the astrocyte cultures after treatment with resveratrol. Cells were cultured in DMEM/10% SFB to confluence and then transferred to serum-free DMEM, incubated for 24 h with resveratrol and fixed with paraformaldehyde and immuno-stained with anti-GFAP as described in Methods section. Representative images showed phase-contrast (panels **a**, **c**, and **e**) and immunocytochemistry for GFAP (panels **b**, **d**, and **f**) from control (0.25% ethanol), 25 and 250 μM resveratrol, respectively. All images are representative fields from at least three independent experiments carried out in triplicate. Scale bar = 50 μm

Resveratrol and glutamate uptake

Basal glutamate uptake under our conditions was about 3.15 nmol/mg protein/min and was not affected by 0.25% ethanol (Fig. 2). Treatment of astrocytes with 25 μM resveratrol for 24 h was able to induce a significant increase in glutamate uptake. Conversely, incubation of astrocytes with a 10-fold higher concentration of resveratrol leads to a significant decrease in glutamate uptake.

Resveratrol and glutamine synthetase

In order to investigate the effect of resveratrol on glutamine synthetase, we measured its activity after 24 h of treatment with resveratrol (Fig. 3). Basal glutamine synthetase activity was about 2.37 μmol/mg protein/h. With 250 μM resveratrol, a significant increase in glutamine synthetase activity was detected, but no effect was observed at lower concentrations.

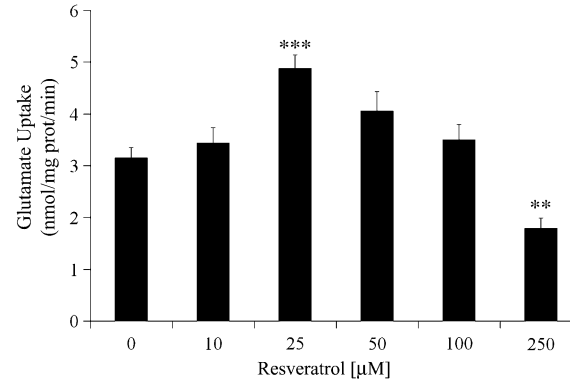

Fig. 2 Effect of resveratrol on glutamate uptake. Cultures of astrocytes were treated for 24 h in the absence or presence of resveratrol at indicated concentrations. Cell culture media were replaced with HBSS and incubated with [^{3}H]-glutamate for 7 min. The data represent the mean ± SEM values from eight independent experiments performed in triplicate. Statistically significant differences from controls (0.25% ethanol), as determined by one-way ANOVA followed by Tukey's multiple variation test, are indicated: $^{**}P < 0.01$ and $^{***}P < 0.001$

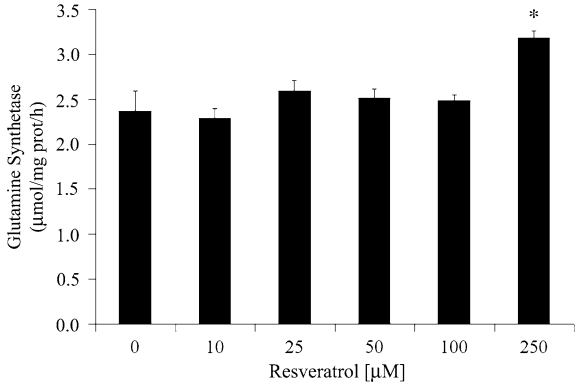

Fig. 3 Influence of resveratrol on glutamine synthetase activity. Cells were treated with increasing concentrations (10, 25, 50, 100, and 250 µM) of resveratrol for 24 h in DMEM without serum. After this time, the incubation medium was removed and GS activity assay was assessed as described in Methods section. The data represent the mean ± SEM values from four independent experiments performed in triplicate. Statistically significant differences from controls (0.25% ethanol), as determined by one-way ANOVA followed by Tukey's multiple variation test, are indicated: $*P < 0.05$

Resveratrol and glutathione content

Next, we examined the effect of resveratrol on glutathione content (Fig. 4). A marked increase in glutathione content was observed when the cells were treated with 25 and 50 µM of resveratrol. Interestingly, resveratrol at a higher concentration (250 µM) tended to decrease glutathione content, but this effect did not reach significance.

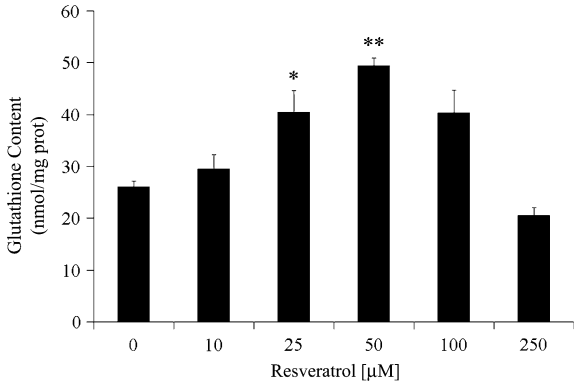

Fig. 4 Effect of resveratrol on glutathione content. After confluence, cells were treated with DMEM without serum in the absence or presence of 10, 25, 50, 100, and 250 µM of resveratrol for 24 h. The data represent the mean ± SEM values of four independent experiments performed in triplicate. Statistically significant differences from controls (0.25% ethanol), as determined by one-way ANOVA followed by Tukey's multiple variation test, are indicated: $*P < 0.05$ and $**P < 0.01$

Resveratrol and S100B secretion

At 24 h of treatment with resveratrol, 50, 100, and 250 µM increased S100B secretion (Fig. 5A). Interestingly, 250 µM resveratrol induced S100B secretion at 6 h and a tendency for increase was observed

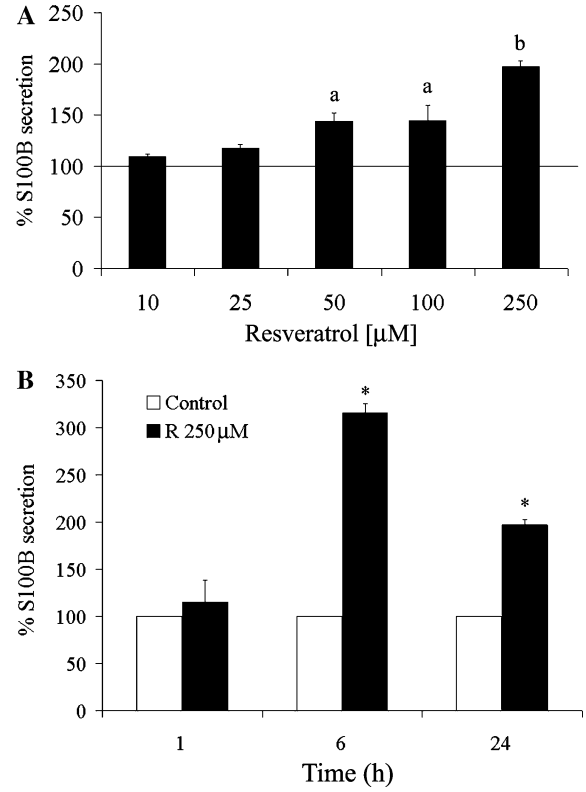

Fig. 5 Effect of resveratrol on S100B secretion in astrocyte culture. In **A**, extracellular S100B was measured by ELISA 24 h after treatment with resveratrol at indicated concentrations in DMEM without serum. Each value is the percentage mean ± SEM from four independent experiments performed in triplicate. Basal secretion (assumed as being 100%) is represented by the continuous line. Different letters indicate statistical difference of extracellular S100B levels from control and other resveratrol concentrations, determined by one-way ANOVA followed by Tukey's multiple variation test, for $P < 0.05$. In **B**, time curve of extracellular S100B in astrocytes exposed to resveratrol (R 250 µM) compared to control in each time. Each value is the percentage mean ± SEM from four independent experiments performed in triplicate. *Significantly different from respective control (Student *t*-test, $P < 0.01$)

during the first hour of exposure of the compound (Fig. 5B).

Discussion

Our data indicate that 24 h exposure to resveratrol was able to modulate basal glutamate uptake in astrocytes. Resveratrol has been shown to be effective against ischemic brain injury and kainic acid-induced seizures or neuronal cell damage in rodents (Wang et al. 2002, 2004). This effect could be related, at least in part, to this effect observed in cultured astrocytes. Glutamate uptake was also increased by resveratrol in C6 glioma cells (Dos Santos et al. 2006).

The neuroprotection imparted by resveratrol has been mainly attributed to its intrinsic antioxidant properties. In vitro studies indicate that glutamate uptake by glial cells is regulated by the surrounding redox environment and that this uptake activity decreases under oxidizing conditions (Trotti et al. 1998). Thus, resveratrol could affect the redox environment of glutamate transporters and favor their activities. Resveratrol, however, is able to induce some cell activities independently of its antioxidant properties (Ovesna and Horvathova-Kozics 2005).

Many antioxidants have anti-inflammatory activity, and resveratrol in particular, due to its structural resemblance with estrogens could provide anti-inflammatory activity by binding to estrogen receptors (Jannin et al. 2004). Moreover, resveratrol has a modulatory activity on cyclooxygenases and nitric oxide synthase (Signorelli and Ghidoni 2005; Kim et al. 2006). Other possible enzyme targets to explain long-term changes induced by resveratrol, for example on glutamate uptake, would be the protein kinases particularly phorbol ester-responsive kinase and MAP kinases (Stewart et al. 2000; Han et al. 2004; Klinge et al. 2005; Guillet et al. 2005). However, the mechanism(s) underlying the long-term changes, particularly in brain need to be clarified.

Astrocytes are the only cells in brain that have the important ability to convert glutamate into glutamine via GS. Glutamine is released to neurons and used for the synthesis of glutamate (and then GABA, in GABAergic neurons). However, glutamate has another important fate in astrocytes, particularly glutathione synthesis (Dringen 2000). Glutamate serves as a substrate per se for glutathione synthesis and as a moiety for exchange by cysteine, another substrate for glutathione synthesis. Interestingly, we did not observe any change in GS activity. Moreover, in concomitantly with the increase in glutamate uptake, an increase in the content of glutathione was seen, reinforcing the antioxidant activity of astrocytes. This effect may also contribute to explain the neuroprotective effect of resveratrol in disorders associated with excitotoxicity and/or oxidative stress. Conversely, astrocytes exposed to a high concentration of resveratrol did not exhibit any increment in glutathione content and, unexpectedly, exhibited an increase in GS activity. Data demonstrating decreased glutamate uptake reinforce the concept of resveratrol as a pro-oxidant compound at elevated concentrations. The effect of elevated resveratrol on GS was surprising, since we observed a decrease in C6 glioma cells (Dos Santos et al. 2006) and many reports describe sensitivity of this enzyme to oxidative stress.

Morphological changes of astrocytes in culture, particularly stellation have been used as a parameter of cell activation (Cechin et al. 2002). For example, adrenaline stimulation of astrocytes (via cAMP) induces cell stellation. Cell stellation in culture has also been observed in stress conditions triggered by many signals, such as beta-hydroxy-butyrate (Leite et al. 2004), ammonia (Leite et al. 2006), and changes in intracellular pH (Cechin et al. 2002). At 250 μM resveratrol, but not at lower concentrations, we observed cell stellation by phase-contrast microscopy and this was confirmed by GFAP immunocytochemistry. Decreased glutamate uptake observed together with cell stellation, at this concentration of resveratrol, indicate strong astrocyte impairment, possibly mediated by the pro-oxidant effect of resveratrol at this elevated concentration.

S100B is a calcium-binding protein expressed and secreted in the central nervous system by astroglia. Besides its intracellular role, S100B works as a cytokine for neighboring cells (astrocytes, neurons, and microglia) depending on its concentration, being neurotrophic at nanomolar levels and apoptotic at micromolar levels (Donato 2001; Van Eldik and Wainwright 2003). We found that resveratrol exposure, 24 h afterwards, increased extracellular S100B in a concentration-dependent manner. Since no parallel loss of LDH was observed at 250 μM

resveratrol, it would be reasonable to conceive that resveratrol affects the mechanism of S100B secretion in astrocytes. Similarly, we found an increase in extracellular S100B in C6 glioma cultures, 24 h afterwards at concentrations of 100 and 250 μM (Dos Santos et al. 2006). Recently, we proposed a negative correlation between glutamate uptake activity and S100B secretion (Tramontina et al. 2006) and our current data reinforce this idea. Moreover, S100B at nanomolar levels appears to protect neurons against glutamate toxicity (see Donato 2001; Van Eldik and Wainwright 2003 for a review). Conversely, persistent and elevated extracellular levels of protein have been associated with neurodegenerative disorders. Thus, it is possible that resveratrol stimulates S100B secretion, in turn stimulating neuronal survival and activity during brain injury and recovery; however, high levels of resveratrol could result in neuronal apoptosis and brain impairment.

Finally, it is important to emphasize that we have investigated resveratrol at concentration ranges from 10 to 250 μM. These values were chosen based on many other recent studies (Wang et al. 2003); however, there is an apparent discrepancy between the concentrations used for in vitro resveratrol efficacy (commonly 5–100 μM) and the extracellular concentrations (less than 2 μM in plasma) (Gescher and Steward 2003). Low levels of resveratrol are due to its fast metabolism in liver and intestinal epithelial cells, mainly due to resveratrol-glucuronide and resveratrol-sulfate (see Signorelli and Ghidoni 2005 for a review). Regardless of this limitation, this study indicated some putative molecular targets of resveratrol in the nervous system, particularly is astrocytes. Other important feature observed is the dual effect of resveratrol on glutamate uptake, depending on its concentration. This also been observed in other biological parameters that are altered by resveratrol, including anti- and pro-oxidant activity and anti- and pro-apoptotic effects (Pervaiz 2004).

In summary, we demonstrate that the neuroprotective activity of resveratrol involves astrocyte activation, as indicated by the in vitro increase of glutamate uptake, glutathione content, and S100B secretion. Our findings reinforce the protective role of this compound in some brain disorders, particularly those involving glutamate toxicity. However, the underlying mechanisms of these changes are not clear at the moment and it is necessary caution with its administration because elevated levels of this compound could contribute to aggravate these conditions.

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