Resveratrol Protects C6 Astrocyte Cell Line against Hydrogen Peroxide-Induced Oxidative Stress through Heme Oxygenase 1

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Abstract

Resveratrol, a polyphenol presents in grapes and wine, displays antioxidant and anti-inflammatory properties and cytoprotective effects in brain pathologies associated to oxidative stress and neurodegeneration. In previous work, we have demonstrated that resveratrol exerts neuroglial modulation, improving glial functions, mainly related to glutamate metabolism. Astrocytes are a major class of glial cells and regulate neurotransmitter systems, synaptic processing, energy metabolism and defense against oxidative stress. This study sought to determine the protective effect of resveratrol against hydrogen peroxide (H₂O₂)-induced cytotoxicity in C6 astrocyte cell line, an astrocytic lineage, on neurochemical parameters and their cellular and biochemical mechanisms. H₂O₂ exposure increased oxidative-nitrosative stress, iNOS expression, cytokine proinflammatory release (TNFα levels) and mitochondrial membrane potential dysfunction and decreased antioxidant defenses, such as SOD, CAT and creatine kinase activity. Resveratrol strongly prevented C6 cells from H₂O₂-induced toxicity by modulating glial, oxidative and inflammatory responses. Resveratrol per se increased heme oxygenase 1 (HO1) expression and extracellular GSH content. In addition, HO1 signaling pathway is involved in the protective effect of resveratrol against H₂O₂-induced oxidative damage in astroglial cells. Taken together, these results show that resveratrol represents an important mechanism for protection of glial cells against oxidative stress.

Introduction

The polyphenol resveratrol (3,5,4′-trihydroxy-trans-stilbene), a redox active compound, is a phytoalexin found in a wide variety of dietary sources including grapes, peanuts and wines, especially red wines [1,2,3,4,5]. Resveratrol presents important antioxidant properties, possibly by its direct scavenging effect and/or activation of pathways those upregulate natural antioxidant defenses [6,7]. Many studies have shown that resveratrol can prevent or slow the progression of a wide variety of illnesses, including cancer [8] and cardiovascular diseases [9,10] and improves health and survival of mice on a high-calorie diet [11]. Moreover, it has been demonstrated that resveratrol has beneficial effects in neurological diseases [6,12,13,14] and is able to inhibit β-amyloid peptide neurotoxicity [15,16]. Whilst direct protective effects of resveratrol against oxidative stress have been demonstrated in neuroglial cells [14,17,18,19,20,21,22], the mechanisms of these effects are not fully understood.

Reactive oxygen species (ROS), such as hydrogen peroxide (H₂O₂), are generated during normal cellular metabolism, playing important roles in signaling pathways [23,24]. However, H₂O₂ also presents toxicological effects, because it can produce new radicals and induces damage to major cellular constituents [25,26,27]. Moreover, it has been demonstrated that exogenous H₂O₂ promotes an imbalance between production and removal of ROS towards the pro-oxidative state, often referred to as oxidative stress.

Heme oxygenase (HO) is the rate-limiting enzyme in the degradation pathway of pro-oxidant heme into biliverdin/bilirubin (both known antioxidants) and has two isoforms: the inducible HO1 and the constitutive HO2 [28]. In the central nervous system (CNS), HO pathway has been reported to be active and to operate as a fundamental defensive mechanism for cells exposed to an oxidant challenge [29]. Increases in HO1 protein levels are associated to protection against stress conditions, such as oxidative stress and hypoxia [30]. HO1 is able to inhibit inducible nitric oxide synthase (iNOS) activity [31], an isoform of nitric oxide synthase that catalyze the synthesis of nitric oxide (NO) from L-arginine. NO plays a physiological role in neuronal cell signaling, but, when in elevated levels, NO becomes toxic, can interact with superoxide anion (generated by mitochondria or by other mechanisms) leading to the overproduction of powerful...
oxidant species peroxynitrite [32]. This toxic compound belongs to a family known as reactive nitrogen species or RNS, which cause cellular damage [33,34]. It has been frequently suggested that polyphenols help to regulate the ROS/RNS imbalance [29,35,36] with potential effect on neuroglial plasticity modulation, including astrocytes activity regulation [14].

Astrocytes have been shown to be involved in the regulation of the brain microenvironment, in particular regarding neurotransmitter systems and ionic homeostasis, synaptic transmission, metabolic support, free-radical scavenging, maintenance of the blood-brain barrier and immune function [37,38,39]. Astrocytes express numerous receptors that enable them to respond to various neuroactive compounds, including neurotransmitters, neuropeptides, growth factors, cytokines, small molecules and toxins [40]. The C6 astrocyte cell line [90% GFAP (glial fibrillary acidic protein) positive after 100 passages] are widely used as an astrocyte-like cell line to study astrocytic function, e.g. glutamate uptake, glutamine synthetase activity, S100B secretion and parameters of oxidative stress. Moreover, this cell line responds quickly to external stimuli, such as H2O2, which can generate oxidative-nitrosative stress [17,41].

Recently, our group has reported that resveratrol was able to modulate important glial parameters involved in brain plasticity and prevents lipid peroxidation, DNA damage and genotoxicity induced by H2O2 exposure in astroglial cells [14,17,41]. Thus, in the present study, we investigated the effect of resveratrol against H2O2-induced toxicity in C6 astrocyte cell line on neurochemical parameters and their cellular and biochemical mechanisms. Nitrite production; the expression of iNOS and HO1 proteins; intracellular ROS production; total antioxidant reactivity (TAR) levels; mitochondrial membrane potential and creatine kinase (CK) activity were assessed. The activity of the main antioxidant enzymes: superoxide dismutase (SOD – EC 1.15.1.1), catalase (CAT – EC 1.11.1.6) and glutathione peroxidase (GPx – EC 1.11.1.9); the extracellular glutathione (GSH) levels and tumor necrosis factor α (TNFα) levels were also evaluated. Additionally, the mechanism of the protective effect of resveratrol against H2O2-oxidative insult was also explored.

Materials and Methods

Materials

Resveratrol, chemical reagents, anti-iNOS and cell culture materials were purchased from Sigma (St. Louis, MO, USA), except for Dulbecco’s Eagle’s medium (DMEM), fetal bovine serum (FBS) and JC-1, which were purchased from Gibco/Invitrogen (Carlsbad, CA, USA). Anti-HO1 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). RANSOD was purchased from Randox (Crumlin, CA, UK). All other chemicals were purchased from local commercial suppliers.

Maintenance of Cell Culture

The C6 astrocyte cell line were obtained from the American Type Culture Collection (Rockville, MD, USA) and were maintained essentially according to previously described [42]. The cells were seeded in flasks and cultured in DMEM (pH 7.4) containing 5% FBS, 2.5 mg/mL Fungizone® and 100 U/mL gentamicin. Cells were kept at a temperature of 37°C in an atmosphere of 5% CO2/95% air. Exponentially growing cells were detached from the culture flasks using 0.05% trypsin/ethylene-diaminetetraacetic acid (EDTA) and seeded 10^5 cells/cm² in 96-, 24- or 6-well plates.

Resveratrol Protects C6 Astrocyte Cell Line

Resveratrol and Hydrogen Peroxide Treatment

After cells reached confluence, the culture medium was removed from well plates and cells were pre-incubated in the absence or presence of 100 μM of resveratrol for 1 h, in serum-free DMEM (pH 7.4). After pre-incubation, resveratrol was maintained and 1 mM H2O2 was added for 0.5 h. During all procedure, cells were maintained at 37°C in an atmosphere of 5% CO2/95% air. Control cells were exposed to 0.25% ethanol vehicle. For all parameters analyzed, the results obtained with vehicle were not different from those obtained under basal conditions without ethanol.

Nitric Oxide Production

Nitric oxide was determined by measurement of nitrite (a stable oxidation product of NO), based on 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 deionized water. The assay was performed as described [43], with modifications. Briefly, cells were cultured on 96-well plate and after treatment, the Griess reagent was added directly to the cell culture and the incubation was maintained under reduced light at room temperature during 15 min. Samples were analyzed at 550 nm on a microplate spectrophotometer. Controls and blanks were run simultaneously. Nitrite concentrations were calculated using a standard curve prepared with sodium nitrite (0–50 μM).

Western Blot Analysis

Cells were removed from plates after treatments using lysis solution with 4% SDS, 2 mM EDTA, 50 mM Tris-HCl, pH 6.8. Equal amounts of proteins from each sample were boiled in sample buffer [62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 5% β-mercaptoethanol, 10% (v/v) glycerol, 0.002% (w/v) bromophenol blue] and submitted to electrophoresis in 10% (w/v) SDS-polyacrylamide gel. The separated proteins were blotted onto a nitrocellulose membrane. Equal loading of each sample was confirmed with Ponceau S staining (Sigma). The following polyclonal antibodies were used: anti-iNOS (1:10000; Sigma) and anti-HO1 (1:3000; Santa Cruz). β-actin was used as a loading control. After incubating overnight with the primary antibody at room temperature, membranes were washed and incubated with peroxidase-conjugated anti-rabbit immunoglobulin (IgG) at a dilution of 1:1000 for 1 h. The chemiluminescence signal was detected using an ECL (Amersham), after the films were scanned and bands were quantified using the Scion Image software.

Intracellular ROS Production

DCFH oxidation was used to measure intracellular ROS production. DCFH-DA (2′,7′-dichlorodihydrofluorescein diacetate) is hydrolyzed by intracellular esterases to dichlorofluorescein (DCFH), which is trapped within the cell. This non-fluorescent molecule is then oxidized to fluorescent dichlorofluorescein (DCF) by action of cellular oxidants. To test whether HO1 was involved in the effect of resveratrol against H2O2-induced increase ROS production, we used its specific inhibitor, Zint Protoporphyrin IX (ZnPp IX) [40] for 0.5 h before the treatment described for resveratrol and H2O2. After, cells were treated with DCFH-DA (10 μM) for 0.5 h at 37°C. Following DCFH-DA exposure, the cells were 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 [17]. The ROS production was
calculated as Unit of Fluorescence – UF/mg protein and was expressed as percentage of control.

**Total Antioxidant Reactivity (TAR)**

TAR, which represents the reactivity of the tissue antioxidants, was determined by measuring the luminescence intensity induced by 2,2’-azo-bis-[2-amidinopropane] (ABAP) [44]. The background luminescence was measured by adding 2 mM ABAP (in 0.1 M glycine buffer, pH 8.6) into a glass scintillation vial. After, luminescent (4 mM) were added to each vial, and the chemiluminescence was determined. This was considered to be the basal value. Cell homogenates were then added and the chemiluminescence was measured during 60 s. The addition of trolox (calibration curve) or cells supernatants reduce the chemiluminescence and this rapid reduction in luminescence is considered measure of TAR capacity. The ratio between the initial and the final chemiluminescence values was used to calculate TAR. TAR values were calculated as nmol trolox/mg protein and are presented as percentage of control.

**Mitochondrial Membrane Potential (JC-1 assay)**

For determination of the mitochondrial membrane potential, after resveratrol and H$_2$O$_2$ treatments, cells were incubated for 0.5 h with JC-1 (5,5’6,6’-tetrachloro-1,1’,3,3’-tetraethylbenzimidazolylcarbocyanine iodide, 2 µg/ml). After, cells were centrifuged, washed once with HBSS and transferred to a 96-well plate. Fluorescence was measured using excitation and emission wavelengths of 545 and 540/590 nm, respectively. The mitochondrial membrane potential was calculated using the ratio of 590 nm (red fluorescent J-aggregates)/540 nm (green monomeric) [45,46].

**Creatine Kinase (CK) Activity**

Cells were homogenized with a 0.9% saline solution and pre-incubated for 15 min at 37°C in a mixture containing: 7 mM phosphocreatine, 9 mM MgSO$_4$, 60 mM Tris–HCl buffer (pH 7.5). Incubation was started by the addition of 3.2 mM ADP plus 0.8 mM reduced glutathione. The reaction was stopped after 10 min by the addition of 10 µmol p-hydroxymercuribenzoic acid. The reagent concentrations and the incubation time were chosen to assure linearity of the enzymatic reaction. Appropriate controls were carried out to measure chemical hydrolysis of phosphocreatine. The creatine formed was estimated according to the colorimetric method [47]. The color was developed by the addition of 20% p-naphthol and 20% diacetyl and read after 20 min at 540 nm. Results were obtained as µmol of creatine formed/min/mg protein.

**Superoxide Dismutase (SOD) Activity**

SOD activity was determined using the RANSOD kit from Randox (Astrum, UK). The method is based on the formation of red formazan from the reaction of 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride and superoxide radical produced in the incubation medium from the xanthine and xanthine oxidase reaction system, which is assayed spectrophotometrically at 505 nm. The inhibition of the produced chromogen is proportional to the activity of the SOD present in the sample. A 50% inhibition is defined as one unit of SOD and the specific activity is represented as U/mg protein.

**Catalase (CAT) Activity**

CAT activity was assayed by the method of Aebi [48], by measuring the absorbance decrease at 240 nm in a reaction medium containing 20 mM H$_2$O$_2$, 0.1% Triton X-100, 10 mM potassium phosphate buffer, pH 7.0 and 50 µg protein. One unit (U) of the enzyme is defined as 1 µmol of H$_2$O$_2$ consumed per minute and the specific activity is reported as U/mg protein.

**Glutathione Peroxidase (GPx) Activity**

GPx activity was measured by the method of Wendel [49], using tert-buty1-hydroperoxide as substrate. GPx activity was determined by monitoring NADPH (0.1 mM) disappearance at 340 nm in a medium containing 2 mM GSH, 0.15 U/ml glutathione reductase, 0.4 mM azide and 0.5 mM tert-buty1-hydroperoxide. One GPx unit is defined as 1 µmol of NADPH consumed per minute and the specific activity is represented as U/mg protein.

**Extracellular Glutathione (GSH)**

Extracellular GSH levels were measured according to Browne and Armstrong with modifications [17,50]. We also examined the effect of HO1 inhibitor on extracellular GSH levels, incubating Zinc Protoporphyrin IX (ZnPP IX) 10 µM, for 0.5 h before the treatment described for resveratrol and H$_2$O$_2$. Extracellular medium were diluted in 100 mM sodium phosphate buffer, pH 8.0, containing 5 mM EDTA, and protein was precipitated with 1.7% meta-phosphoric acid. After centrifugation, supernatant was assayed with o-phthalaldehyde (1 mg/ml) methanol at room temperature 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 (0–500 µM) and the concentrations were calculated as nmol/mg of protein. The results were expressed as percentage of control.

**Tumor Necrosis Factor α (TNF-α) Levels**

This assay was carried out in extracellular medium, using a rat TNF-α ELISA from R&D Systems (USA). To test whether the effect of resveratrol against H$_2$O$_2$-induced TNF-α levels was through HO1, we used a specific inhibitor, Zinc Protoporphyrin IX (ZnPP IX) 10 µM, for 0.5 h before the treatment described for resveratrol and H$_2$O$_2$.

**Protein Determination**

Protein content was measured by Lowry’s method using bovine serum albumin as standard [51].

**Statistical Analysis**

All quantitative data and experiments described in this study were repeated at least three times. Results are expressed as mean ± S.E.M. Differences between groups were analyzed statistically by two way analysis of variance (ANOVA) followed by the Tukeys test using SPSS (Statistical Package for the Social Sciences software, version 16.0 for Windows). Values of P<0.05 were considered statistically significant.

**Results**

**Resveratrol Decreased Nitrite Levels**

The production of NO was indirectly measured by the formation of nitrite, expressed in µM. Resveratrol per se decreased nitrite production, from 13±0.9 to 11±0.7 µM, compared to the control conditions (Fig. 1). H$_2$O$_2$ increased nitrite formation up to 16% (from 13±0.9 to 15.1±0.7 µM). This effect was completely prevented by resveratrol. To test whether decreased in nitrite accumulation induced by resveratrol was dependent on NOS activity, we examined the effect of resveratrol in the presence of...
the L-NAME (N\textsuperscript{\textdegree}-nitro-L-arginine methyl ester), a NOS inhibitor. The co-incubation of resveratrol with L-NAME decreased the nitrite levels up to 22% compared to the basal value (Fig. 1).

Resveratrol Prevented H\textsubscript{2}O\textsubscript{2}-induced iNOS Expression
Considering that the excess production of NO, generated primarily by iNOS, has been implicated as a mediator of cellular injury, we aimed to determine the effect of resveratrol on iNOS expression in the presence of H\textsubscript{2}O\textsubscript{2}. As expected, H\textsubscript{2}O\textsubscript{2} increased iNOS immunoreactivity (about 50%) compared with the control conditions (Fig. 2). This effect was inhibited by resveratrol to values near to basal levels, indicating that resveratrol could effectively suppress iNOS expression, which was stimulated by H\textsubscript{2}O\textsubscript{2}.

Resveratrol Enhanced HO\textsubscript{1} Expression
Increase in HO\textsubscript{1} protein levels are associated to protection against stress conditions, such as oxidative stress, hypoxia and neurodegenerative disorders [7]. Then we evaluated the immunoreactivity of this enzyme. Fig. 3 shows that resveratrol \textit{per se} and under oxidative insult was able to increase the expression of HO\textsubscript{1} (by about 40% and 30%, respectively) in C6 astrocyte cell line. H\textsubscript{2}O\textsubscript{2} did not affect the HO\textsubscript{1} expression.

Resveratrol Reduced Intracellular ROS Production through HO\textsubscript{1}
As an imbalance between production and removal of ROS toward the pro-oxidative state can be promoted by the cellular exposure to H\textsubscript{2}O\textsubscript{2}, we investigated the effect of resveratrol and resveratrol combined with HO\textsubscript{1} inhibitor on intracellular ROS production by DCFH oxidation method (Fig. 4). Resveratrol \textit{per se} decreased by about 16% the DCFH oxidation compared to control conditions. In addition, resveratrol was able to prevent the increase of ROS production induced by H\textsubscript{2}O\textsubscript{2}. When cells were pre-incubated with ZnPP IX (10 \textmu M) resveratrol partially decreased DCFH oxidation and did not prevent the effect of H\textsubscript{2}O\textsubscript{2} exposure, indicating that antioxidant effect of resveratrol probably was through HO\textsubscript{1}. Interestingly, under HO\textsubscript{1} inhibitor, H\textsubscript{2}O\textsubscript{2} potentiated the increase in ROS levels.

Resveratrol Increased Total Antioxidant Reactivity (TAR)
Resveratrol increased (50%) TAR levels compared to control conditions (Fig. 5). Under oxidative insult the TAR levels were reduced by about 15% compared to basal value. This effect was completely prevented by resveratrol, which increased TAR levels up to 55%, compared to H\textsubscript{2}O\textsubscript{2}-oxidative insult.

Resveratrol Prevented Mitochondrial Dysfunction
The mitochondria have emerged as key regulators of oxidative stress and cytotoxicity [32,52]. As expected H\textsubscript{2}O\textsubscript{2} reduced the mitochondrial membrane potential by about 25%, inducing mitochondrial dysfunction and resveratrol completely prevented this effect (Fig. 6). Resveratrol \textit{per se} did not affect the mitochondrial membrane potential.

Resveratrol Prevented the Impairment in CK Activity Induced by H\textsubscript{2}O\textsubscript{2}
The enzyme CK is a target of ROS/RNS and we evaluated the effect of resveratrol on the CK activity in the presence or absence of H\textsubscript{2}O\textsubscript{2} (Fig. 7). As expected, oxidative insult decreased CK activity by 15% (from 1.5\pm0.1 to 1.3\pm0.1) and resveratrol was able to completely prevent this effect. Resveratrol \textit{per se} did not affect CK activity.
Effects of Resveratrol on Superoxide Dismutase (SOD), Catalase (CAT) and Glutathione Peroxidase (GPx) Activity

In order to determine the effect of resveratrol on antioxidant cellular defenses, we studied the activities of SOD, CAT and GPx (Fig. 8). Resveratrol was able to increase only the activity of SOD (from 2.7 ± 0.1 to 3.1 ± 0.1 U/mg protein) compared to control conditions (Fig. 8A). The H2O2-induced oxidative stress decreased SOD (to 1.9 ± 0.1 U/mg protein) (Fig. 8A) and CAT (from 14.2 ± 1.3 to 12.8 ± 1.1 U/mg protein) (Fig. 8B) activities, and these effects were prevented by resveratrol. Resveratrol and H2O2 did not affect GPx activity (Fig. 8C). However, resveratrol decreased GPx activity (20%) under H2O2 exposure.

Resveratrol Increased Extracellular GSH Levels

Resveratrol per se is able to modulate GSH, the main antioxidant of CNS, increasing intracellular GSH content in C6 cells [17]. Here, we demonstrated that resveratrol increased extracellular GSH levels, by about 13%, compared to the control conditions (Table 1). Resveratrol also increased GSH extracellular content after the H2O2 exposure. To verify the effect of resveratrol on extracellular GSH was dependent of HO1, we pre-treated the cells with ZnPP IX. HO1 inhibitor prevented the increase of extracellular GSH induced by resveratrol.

Resveratrol Prevented the H2O2-stimulated TNFα Levels

To determine whether resveratrol could modulate the pro-inflammatory cytokines under H2O2 exposure, we measured the TNFα levels. Resveratrol per se did not affect the levels of TNFα, but was able to completely prevent the augment induced by H2O2 exposure, decreasing the levels of this cytokine, from 130 ± 10 to 99 ± 8 (Table 1). We examined whether the effect of resveratrol was dependent of HO1 and pre-treated the cells with HO1 inhibitor.
The effect of resveratrol in the brain has been studied in a variety of pathological events, including carcinogenesis [53], ischemic injury [54] and neurodegenerative disorders [15,16,55]. However, the cellular mechanisms underlying resveratrol-induced selective protection to the neural system needs to be better elucidated. The present data shows that resveratrol is able to modulate important antioxidant defenses in C6 astrocyte cell line.

High concentrations of H2O2 induce oxidative-nitrosative stress, which can lead to lipid, protein and DNA oxidation, causing cellular damage [56,57]. It is important to emphasize that in this study H2O2 did not induce cell death, in agreement with other studies of our group [17,41,58]. Resveratrol decreased NO levels and RNS (possibly peroxynitrite) implicated in the pathogenesis of neurodegenerative disorders like Alzheimer’s and Parkinson’s diseases [34]. Peroxynitrite, resulted by the reaction between NO and superoxide anion, is one of the main molecules responsible for the cellular damage in neurodegenerative disorders [34].

Oxidative stress upregulates the expression of iNOS and presumably would lead to increase in the NO concentrations [16]. NO is synthesized during the stoichiometric conversion of L-arginine to L-citrulline in the presence of oxygen and nicotinamide adenine dinucleotide phosphate (NADPH), which is catalyzed by NOS [39]. Our results have indicated that resveratrol decreases iNOS expression. Previous works already demonstrated that resveratrol may act through iNOS [16,60,61]. Thus, these results suggest that resveratrol may have a protective effect against diseases associated with increase in iNOS synthesis and NO levels.

Resveratrol, when used as a selective near-infrared (near-IR) contrast agent, was also able to modulate the activity of HO1. Sakata et al., 2010 [30] showed that HO1 might be a unique candidate by which resveratrol can induce an endogenous cellular pathway which leads to building cellular and/or organ resistance to stress, indicating a neuroprotective effect. In agreement with Sakata, we showed that resveratrol, via HO1, has antioxidant effects and thus could explain the actions of resveratrol in CNS [7]. Furthermore, there is a relationship between HO1 and iNOS. The nuclear factor-erythroid-2-related factor 2 (Nrf2) regulates the transcription of HO1, which acts as a scavenger of NO [31]. Nrf2 also mediates neuroprotection and modulates several detoxification genes that encode antioxidant proteins, such as GSH system and thioredoxin, regulators of intracellular redox environment [61,62,63,64]. Resveratrol activates Nrf2 and all these regulators [61,64,65,66,67,68,69,70]. Excess of NO acts as a positive signal to increase HO1, which in turn, is able to scavenge NO and block the activity of iNOS, to prevent further NO production. Resveratrol, under oxidative conditions, induced a decrease in iNOS and an increase in HO1 immunocontent. Thus, HO1 can also be critical to signaling antioxidant response of resveratrol.

Besides, resveratrol decreased DCFH oxidation and prevented H2O2-oxidative insult. This suggests strong antioxidant properties for this polyphenol [71]. Excessive free radical, such as peroxynitrite, can lead to lipid, protein and DNA oxidation; and these events are associated to age-related diseases [16,72]. Our group has been demonstrated that resveratrol modulates glutamate metabolism and protects against genotoxicity, probably by their scavenger properties and astrocyte activation [17,41]. Resveratrol under oxidative insult inhibited iNOS and decreased DCFH oxidation, emerging as an important molecule which provides protection against cellular toxicity. Our data on intracellular ROS production also indicated the involvement of HO1 signaling pathway in the protective mechanism of resveratrol against H2O2-induced oxidative stress.

TAR levels represent the antioxidant capacity of cells and resveratrol was able to increase TAR levels by about 50%. This suggests that resveratrol displays antioxidant effects, protecting cells against oxidative damage. It might avoid free radical generation and improve antioxidant defenses, such as GSH.
H2O2 decreased TAR levels. This effect elicited by resveratrol has been mainly attributed to its intrinsic antioxidant properties. However, it is important to mention that it is also able to modulate diverse cell activities independently of its antioxidant properties [76].

The mitochondrial membrane potential has been implicated as a factor in impaired mitochondrial function [77]. The impairment in this organelle have been shown to occur in various models of cell injury [32]. A decrease in mitochondrial membrane potential following an intense production of ROS and RNS induces mitochondrial disruption, inhibition of mitochondrial respiratory chain, reduction in ATP synthesis and cell death [78]. In this sense, in our study H2O2 generates high levels of ROS/RNS that induce mitochondrial membrane potential dysfunction. Resveratrol totally prevent this damage. In addition, brain cells contain antioxidative defense mechanisms that can protect against oxidative damage and resveratrol may be a key regulator of these defenses.

We also showed that although resveratrol did not modify CK activity per se, it was able to prevent H2O2-induced CK activity decrease. CK consists of a family of enzymes involved in high-energy consuming tissues such as brain and skeletal muscle and it is also very sensitive to oxidative damage (oxidation and nitration). Considering that CK contains in its active site sulphhydryl group extremely important for their full operation, it is proposed that reduced activity is related to increase in ROS/RNS production induced by H2O2, which could oxidize thiol groups, contributing to CK activity inhibition [79]. The marked reduction of CK activity is reported in the brain of patients with oxidative-stress linked neurodegenerative diseases, such as Alzheimer’s and Parkinson’s diseases [79]. This indicates that resveratrol can display in vivo function on CK activity in pathologies related to redox imbalance.

The levels of free radicals can be determined by the balance

![Figure 8. Effects of resveratrol on SOD, CAT and GPx activity.](image)

Cells were pre-treated for 1 h in the presence of 100 μM resveratrol (RSV) followed by the addition of 1 mM H2O2 for 0.5 h. SOD (A), CAT (B) and GPx (C) activities were measured as described in the Experimental procedures section. All data represent mean ± S.E.M. of three independent experimental determinations performed in triplicate, analyzed statistically by two-way ANOVA followed by the Tukey’s test.

(a) indicates significant differences from control (P<0.05). (b) indicates significant differences from H2O2 (P<0.05).

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Table 1. Effect of resveratrol on extracellular GSH content and TNFα levels.

| Treatments | Extracellular GSH | TNFα |
|------------|------------------|------|
| RSV        | 113±6 (a)        | 101±10 |
| H2O2       | 90±10            | 130±10 (a) |
| RSV+H2O2   | 111±7 (a, b)     | 99±8 (b) |
| ZnP IX     | 98±6             | 98±9 (b) |
| RSV+ZnP IX | 102±8 (b)        | 97±8 (b) |
| H2O2+ ZnP IX | 88±10 (a)    | 127±12 (a, c) |
| RSV+H2O2+ ZnP IX | 87±8 (a, c) | 125±12 (a, c) |

Cells were pre-treated for 1 h in the presence of 100 μM resveratrol (RSV) followed by the addition of 1 mM H2O2 for 0.5 h. Cells were also pre-incubated for 0.5 h with ZnP IX (10 μM), a HO1 inhibitor, before the pre-treatment with resveratrol. Extracellular GSH and TNFα levels were measured as described in the Experimental procedures section. Data are expressed as percentage of control value (assumed to be 100%) and represent mean ± S.E.M. of three experimental determinations performed in triplicate, analyzed statistically by two-way ANOVA followed by the Tukey’s test. (a) indicates significant differences from control (P<0.05), (b) indicates significant differences from H2O2 (P<0.05) and (c) indicates significant differences from ZnP IX inhibitor (P<0.05).

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between their rate of production and clearance by various antioxidant compounds and enzymes such as SOD, CAT and GPx. Resveratrol prevented completely the H$_2$O$_2$-effect reestablishing SOD activity, which in turn can decrease superoxide radicals [80,81]. In this context, modulation of SOD activity by resveratrol is very important to the neuroprotective effect associated to oxidative stress. However, we also observed an increase in CAT activity compared to oxidative insult, showing that the H$_2$O$_2$ is decomposed to water [26,82]. Resveratrol decreases the GPx activity under H$_2$O$_2$-induced oxidative stress, improving the levels of GSH, the major brain antioxidant, produced by astrocytes. As many redox-active compounds modulate major enzymatic defenses against free radical action [83,84,85,86], the anti- or pro-oxidant role is determined by the set of their actions and cell types studied [5,35].

We have been showed that resveratrol modulates astroglial parameters related to glutamate metabolism [14,17,42] including the increase in glutamate uptake (probably by increase in EAAC1 expression) and GSH levels. GSH is secreted by astrocytes and serves as a substrate to neuronal GSH synthesis [80,87]. Metal chelators, SOD and antioxidants (e.g. resveratrol) inhibit the oxidation of GSH, when it is secreted by astrocytes [80]. Moreover, the increase in GSH levels in glial cells confers neuroprotection in neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases [27,38]. In this sense, resveratrol increases extracellular GSH levels. The effect of resveratrol on extracellular GSH probably involves the HO1 pathway. In addition, Escartin et al, 2011 [89] reported that Nrf2, the upregulator of HO1, facilitates the glutathione synthesis by regulation of EAAC1, the main glutamate transporter present in C6 astroglial cells. The increase in GSH biosynthesis through Nrf2 also protected glial cells against oxidative damage [90].

Lee et al, 2010 [88] demonstrated that a depletion of GSH in glial cells induces inflammatory response. TNFα plays important roles in ROS production [91] and it also potentiates NO production in astrocytes [92]. Thus, an extracellular stimulus such as H$_2$O$_2$ induces an increase in ROS/RNS production and in TNFα levels. In this sense, resveratrol was able to prevent the proinflammatory cytokine increase induced by H$_2$O$_2$ exposure, probably by HO1 pathway, an upstream signal transduction of nuclear factor-κB (NF-κB) and iNOS expression [31]. Even though its mechanism of action remains not fully understood, resveratrol may exert its effects by antioxidant/scavenger activity, by modulation of NO metabolism and HO1 expression or by anti-inflammatory effect [6,16,29,30,93,94,95,96].

In summary, we demonstrated the cytoprotective effects of resveratrol against oxidative damage in C6 astrocyte cell line. The main conclusions of this study are depicted in Fig. 9, which displays that resveratrol strongly prevented the increase in ROS/RNS production, iNOS expression and TNFα levels induced by H$_2$O$_2$ in C6 astrocyte cell line and the putative involvement of HO1 activation in the protective role of resveratrol. H$_2$O$_2$ exposure induced decrease in mitochondrial membrane potential, TAR levels, SOD, CAT and CK activity and resveratrol was also able to protect glial cells. Moreover, resveratrol per se increased the HO1 expression, the TAR levels, SOD activity and extracellular GSH content and decreased the basal levels of ROS/RNS. Our results suggest that resveratrol modulates important functions related to the maintenance of the cellular redox environment through HO1 signaling pathway. Overall, these observations suggest that resveratrol may potentially be useful for therapeutic purposes as a potent inducer of HO1 for the protection of glial cells against oxidative response.

Author Contributions

Conceived and designed the experiments: AQS LDB AL CAG CG. Performed the experiments: AQS LDB AL CAG CG. Analyzed the data: AQS LDB AL MW DOS CAG CG. Contributed reagents/materials/analysis tools: AQS AL MW DOS CAG CG. Wrote the paper: AQS CAG CG.

References

1. Fremont L (2000) Biological effects of resveratrol. Life Sci 66: 665–673.
2. Soleas GJ, Diamandis EP, Goldberg DM (1997) Resveratrol: a molecule whose time has come? And gone? Clin Biochem 30: 91–113.
3. Pervaiz S (2003) Resveratrol: from grapevines to mammalian biology. FASEB J 17: 1975–1985.
4. Delmas D, Jamin B, Latruffe N (2005) Resveratrol: preventing properties against vascular alterations and ageing. Mol Nutr Food Res 49: 377–395.
5. de la Lastra CA, Villegas I (2007) Resveratrol as an antioxidant and pro-oxidant agent: mechanisms and clinical implications. Biochim Biophys Acta 1756-1160.
6. Baur JA, Sinclair DA (2006) Therapeutic potential of resveratrol: the in vivo evidence. Nat Rev Drug Discov 5: 493–506.
7. Bastianetto S, Quirion R (2010) Heme oxygenase 1: another possible target to explain the neuroprotective action of resveratrol, a multitargeted nutrient-based molecule. Exp Neurol 225: 237–239.
8. Jiang M, Cai L, Udenci GO, Slowing KY, Thomas CF, et al. (1997) Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. Science 273: 218–220.
9. Pervaiz S (2004) Chemotherapeutic potential of the chemopreventive phytoalexin resveratrol. Drug Resist Updat 7: 333–344.
10. Ungvari Z, Labinskyy N, Mukhopadhyay P, Pinto JT, Bagi Z, et al. (2009) Resveratrol attenuates mitochondrial oxidative stress in coronary arterial endothelial cells. Am J Physiol Heart Circ Physiol 297: H1107–1181.
11. Baur JA, Pearson KJ, Price NL, Jamieson HA, Linen C, et al. (2006) Resveratrol improves health and survival of mice on a high-calorie diet. Nature 444: 337–342.
12. Virgil M, Contesta A (2000) Partial neuroprotection of in vivo excitotoxic brain damage by chronic administration of the red wine antioxidant agent, trans-resveratrol in rats. Neurosci Lett 281: 123–126.

13. Sinha K, Chaudhary G, Gupta TK (2002) Protective effect of resveratrol against oxidative stress-induced midbrain cerebral artery occlusion model of stroke in rats. Life Sci 71: 653–665.

14. Quincozes-Santos A, Gottfried C (2011) Resveratrol modulates astrogial functions: neuroprotective hypothesis. Ann N Y Acad Sci 1215: 72–76.

15. Richard T, Pavan DS, Jégou ML, Pedrot E, Waffo-Teguo P, et al. (2011) Neuroprotective properties of resveratrol and derivatives. Ann N Y Acad Sci 1215: 103–108.

16. Huang TC, Lu KT, Wu YY, Wu YJ, Yang YL (2011) Resveratrol protects rats from Aβeta-induced neurotoxicity by the reduction of iNOS expression and lipid peroxidation. PLoS One 6: e29102.

17. Quincozes-Santos A, Nardin P, de Souza DF, Gelain DP, Moreira JC, et al. (2009) The janus face of resveratrol in astrogial cells. Neurotox Res 16: 30–41.

18. Vieira de Almeida LM, Pinheiro CC, Leite MC, Brolese G, Leal RB, et al. (2008) Protective effect of resveratrol on hydrogen peroxide induced toxicity in primary cortical astrocyte cultures. Neurochem Res 33: 8–15.

19. de Almeida LM, Leite MC, Thomazi AP, Battu C, Nardin P, et al. (2008) Resveratrol protects against oxidative injury induced by H2O2 in acute hippocampal slice preparations from Wistar rats. Arch Biochem Biophys 480: 27–32.

20. Fuku M, Choi HJ, Zhu BT (2010) Mechanism for the protective effect of resveratrol against oxidative stress-induced neuronal death. Free Radic Biol Med 49: 800–813.

21. Trasci V, Chopra K (2011) Resveratrol prevents alcohol-induced cognitive deficits and brain damage by blocking inflammatory signaling and cell death cascade in neonatal rat brain. J Neurochem 117: 678–690.

22. Lee EO, Park HJ, Kang JL, Kim HS, Chong YH (2010) Resveratrol reduces glutamate-mediated monocyte chemotactic protein-1 expression via inhibition of extracellular signal-regulated kinase 1/2 pathway in rat hippocampal slice cultures. J Neurochem 112: 1477–1487.

23. Dringen R, Kussmaul I, Hamprecht B (1998) Detoxification of exogenous hydrogen peroxide and organic hydroperoxides by cultured astrogial cells assessed by microtiter plate assay. Brain Res Brain Res Protoc 2: 223–228.

24. Dringen R, Pavlovs PG, Hirrlinger J (2005) Peroxide detoxification by brain cells. J Neurosci Res 79: 157–165.

25. Forman HJ (2007) Use and abuse of exogenous H2O2 in studies of signal transduction. Free Radic Biol Med 42: 296–302.

26. Droge W (2002) Free radicals in the physiological control of cell function. Physiol Rev 82: 47–95.

27. Halliwell B (2006) Oxidative stress and neurodegeneration: where are we now? Trends Pharmacol Sci 27: 231–236.

28. Halliwell B (2009) The workings of a free radical. Free Radic Biol Med 46: 531–542.

29. Gao HM, Zhou H, Hong JS (2012) NADPH oxidases: novel therapeutic targets for neurodegenerative diseases. Trends Pharmacol Sci 33: 295–303.

30. Quincozes-Santos A, Andreazzza A, Nardin P, Funchal G, Gonçalves CA, et al. (2007) Resveratrol attenuates oxidative-induced DNA damage in C6 Gliona cells. Neurotox Res 20: 380–391.

31. Murias M, Jager W, Handler N, Erker T, Horvath Z, et al. (2005) Antioxidant, proapoptotic and prooxidant and cytotoxic activity of hydroxylated resveratrol analogues: therapeutic implications for antioxidant treatment. Drugs Aging 18: 685–716.

32. Calabrese V, Cornelius C, Mancuso C, Pennisi G, Calafato S, et al. (2008) Protective effects of resveratrol on hydrogen peroxide induced toxicity in middle cerebral artery occlusion model of stroke in rats. J Biol Chem 283: 35710–35718.

33. Calabrese V, Delpini D, Calabro P, Murias M, Gouin C, et al. (2008) Mitochondrial membrane potential monitored by JC-1 dye. Methods Mol Biol 404: S6–S20.

34. Jou MJ (2008) Pathophysiological and pharmacological implications of mitochondria-targeted reactive oxygen species generation in astrocytes. Adv Drug Deliv Rev 60: 1512–1526.

35. Vargas MR, Johnson DA, Mancamado P (2008) Therapeutic potential of resveratrol in Alzheimer’s disease. BMC Neurosci 9 Suppl 1: P6.

36. Calabrese V, Delpini D, Calabro P, Murias M, Gouin C, et al. (2008) Protective effects of resveratrol against oxidative stress induced by H2O2 in acute hippocampal slice preparations from Wistar rats. Arch Biochem Biophys 480: 27–32.

37. Markiewicz I, Lukomska B (2006) The role of astrocytes in the physiology and pathology of the central nervous system. Acta Neurobiol Exp (Wars) 66: 343–352.

38. Moncada S, Bolanos JP (2006) Nitric oxide, cell bioenergetics and neurodegeneration. Free Radic Biol Med 40: 2319–2327.

39. Arredondo F, Echeverry C, Abin-Carriquiry JA, Blasina F, Antunez K, et al. (2009) Mitochondrial membrane potential assessed by microtiter plate assay. Brain Res Brain Res Protoc 2: 223–228.

40. Murias M, Jager W, Handler N, Erker T, Horvath Z, et al. (2005) Antioxidant, proapoptotic and prooxidant and cytotoxic activity of hydroxylated resveratrol analogues: therapeutic implications for antioxidant treatment. Drugs Aging 18: 685–716.

41. Quincozes-Santos A, Nardin P, Funchal C, de Almeida LM, Jacques-Silva MC, et al. (2006) Resveratrol increases glutamate uptake and glutamate synthetase activity in C6 gliona cells. Arch Biochem Biophys 453: 161–167.

42. Murias M, Jager W, Handler N, Erker T, Horvath Z, et al. (2005) Antioxidant, proapoptotic and prooxidant and cytotoxic activity of hydroxylated resveratrol analogues: structure-activity relationship. Biochem Pharmac 69: 903–912.

43. Halliwell B (2001) Role of free radicals in the neurodegenerative diseases: therapeutic implications for antioxidant treatment. Drugs Aging 18: 685–716.
73. Latini A, Borba Rosa R, Scassati K, Lletuy S, Bello-Klein A, et al. (2002) 3-Hydroxyglutaric acid induces oxidative stress and decreases the antioxidant defenses in cerebral cortex of young rats. Brain Res 956: 367–373.
74. Halliwell B (2006) Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. Plant Physiol 141: 312–322.
75. Gutteridge JM, Halliwell B (2010) Antioxidants: Molecules, medicines, and myths. Biochem Biophys Res Commun 393: 561–564.
76. Ovesna Z, Hořáčková-Kozík R (2005) Structure-activity relationship of trans-resveratrol and its analogues. Nefrologia 52: 450–455.
77. Bai G, Rama Rao KV, Murthy CR, Panickar KS, Jayakumar AR, et al. (2001) Ammonia induces the mitochondrial permeability transition in primary cultures of rat astrocytes. J Neurosci Res 66: 981–991.
78. Choi IV, Lee SJ, Ju C, Nam W, Kim HC, et al. (2000) Protection by a manganese porphyrin of endogenous peroxynitrite-induced death of glial cells via inhibition of mitochondrial transmembrane potential decrease. Glia 31: 155–164.
79. Beal MF (2000) Energetics in the pathogenesis of neurodegenerative diseases. Trends Neurosci 23: 290–304.
80. Pope SA, Milton R, Heales SJ (2008) Astrocytes protect against copper-catalysed loss of extracellular glutathione. Neurochem Res 33: 1410–1418.
81. Spanier G, Xu H, Xia N, Tobias S, Deng S, et al. (2009) Resveratrol reduces endothelial oxidative stress by modulating the gene expression of superoxide dismutase 1 (SOD1), glutathione peroxidase 1 (GPx1), and NADPH oxidase subunit (Nox4). J Physiol Pharmacol 60 Suppl 4: 111–116.
82. Smith-Pearson PS, Kooskhi M, Spitze DR, Poole LB, Zhao W, et al. (2008) Decreasing peroxiredoxin II expression decreases glutathione, alters cell cycle distribution, and sensitizes glioma cells to ionizing radiation and H2O2. Free Radic Biol Med 45: 1178–1189.
83. Sebai H, Sani M, Yaacoubi MT, Aouani E, Ghanem-Boughanmi N, et al. (2010) Resveratrol, a red wine polyphenol, attenuates lipopolysaccharide-induced oxidative damage and dopamine depletion in rat model of Parkinson’s disease. Brain Res 1328: 139–151.
84. Meng Q, Vralalar CN, Ruan R (2008) Effects of epigallocatechin-3-gallate on mitochondrial integrity and antioxidative enzyme activity in the aging process of human fibroblast. Free Radic Biol Med 44: 1032–1041.
85. Khan MM, Ahmad A, Ishaq T, Khan MB, Hoda MN, et al. (2010) Resveratrol attenuates 6-hydroxydopamine-induced oxidative damage and dopamine depletion in rat model of Parkinson’s disease. Brain Res 1328: 139–151.
86. Lee M, Cho T, Jantaranomti N, Wang YT, McGeer E, et al. (2010) Depletion of GSH in glial cells induces neurotoxicity: relevance to aging and degenerative neurological diseases. FASEB J 24: 2533–2545.
87. Escartín C, Wen SJ, Malgorn C, Auregan G, Berman AE, et al. (2011) Nuclear factor erythroid 2-related factor 2 facilitates neuronal glutathione synthesis by upregulating neuronal excitatory amino acid transporter 3 expression. J Neurosci 31: 7392–7401.
88. Vargas MR, Pehar M, Casissa P, Beckman JS, Barbeito L (2006) Increased glutathione biosynthesis by Nrf2 activation in astrocytes prevents p75NTR-dependent motor neuron apoptosis. J Neurochem 97: 687–696.
89. Shen HM, Pervaiz S (2006) TNF receptor superfamily-induced cell death: redox-dependent execution. FASEB J 20: 1589–1598.
90. Hamby ME, Grap groslin AR, Hewett SJ, Hewett JA (2008) TGF beta 1 and TNF alpha potentiate nitric oxide production in astrocyte cultures by recruiting distinct subpopulations of cells to express NOS-2. Neurochem Int 52: 962–971.
91. Vang O, Alam N, Baile CA, Bur A, Brown K, et al. (2011) What is new for an old molecule? Systematic review and recommendations on the use of resveratrol. PLoS One 6: e19881.
92. Kwon KJ, Kim JN, Kim MK, Lee J, Ignarro LJ, et al. (2011) Melatonin synergistically increases resveratrol-induced heme oxygenase-1 expression through the inhibition of ubiquitin-dependent proteasome pathway: a possible role in neuroprotection. J Pineal Res 50: 110–123.
93. Bobermin LD, Quincozes-Santos A, Guerra MC, Leite MG, Souza DO, et al. (2012) Resveratrol prevents ammonia toxicity in astroglial cells. PLoS One 7: e52164.
94. Yun JM, Chien A, Jalal I, Deva R (2012) Resveratrol up-regulates SIRT1 and inhibits cellular oxidative stress in the diabetic milieu: mechanistic insights. J Nutr Biochem 23: 699–703.