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Resveratrol prevents oxidative stress and inhibition of Na^+K^+-ATPase activity induced by transient global cerebral ischemia in rats

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Abstract

Increased oxidative stress and energy metabolism deficit have been regarded as an important underlying cause for neuronal damage induced by cerebral ischemia/reperfusion (I/R) injury. In this study, we investigated the oxidative mechanisms underlying the neuroprotective effects of resveratrol, a potent polyphenol antioxidant found in grapes, on structural and biochemical abnormalities in rats subjected to global cerebral ischemia. Experimental model of transient global cerebral ischemia was induced in Wistar rats by the four vessel occlusion method for 10 min and followed by different periods of reperfusion. Nissl and fluoro jade C stained indicated extensive neuronal death at 7 days after I/R. These findings were preceded by a rapid increase in the generation of reactive oxygen species (ROS), nitric oxide (NO), lipid peroxidation, as well as by a decrease in Na^+K^+-ATPase activity and disrupted antioxidant defenses (enzymatic and non-enzymatic) in hippocampus and cortex. Administrating resveratrol 7 days prior to ischemia by intraperitoneal injections (30 mg/kg) significantly attenuated neuronal death in both studied structures, as well as decreased the generation of ROS, lipid peroxidation and NO content. Furthermore, resveratrol brought antioxidant and Na^+K^+-ATPase activity in cortex and hippocampus back to normal levels. These results support that resveratrol could be used as a preventive, or therapeutic, agent in global cerebral ischemia and suggest that scavenging of ROS contributes, at least in part, to resveratrol-induced neuroprotection.

Keywords: Oxidative stress; Free radicals; Cerebral ischemia; Resveratrol; Neuroprotection; Na^+K^+-ATPase

1. Introduction

Cerebral ischemia is caused by a deficiency in blood supply which triggers various pathophysiological changes. Brief periods of global cerebral ischemia result in delayed neuronal death (DND), which occurs 3–7 days after the initial ischemic insult in selective vulnerable brain regions, especially in the cortex and the CA1 subfield of hippocampus [1]. During the reperfusion period after ischemia, increased oxygen supply results in overproduction of reactive oxygen species (ROS), which are involved in the process of cell death. ROS, such as superoxide anions, hydroxyl free radicals, hydrogen peroxide and nitric oxide are produced as a consequence of metabolic reactions and central nervous system activity [2]. These reactive species are directly involved in oxidative damage of cellular macromolecules such as nucleic acids, proteins, and lipids in ischemic tissues, which can lead to cell death [3]. In fact, the release of ROS and increased lipid peroxidation can be detected early after ischemia/reperfusion (I/R), e.g., 1–3 h, an interval in which there was no sign of neuronal death [4,5]. Superoxide and hydroxyl radical cause severe cell membrane damage by inducing lipid peroxidation [6]. Inducible nitric oxide (NO) synthase is up-regulated after I/R, which results in excessive NO production. This excess NO reacts with superoxide to form peroxynitrite, a powerful radical that can induce neuronal death after cerebral ischemia [7]. The enzymatic antioxidant activity of the tissue affected by I/R is particularly important for the primary endogenous defense against the ROS induced injury. Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) are endogenous antioxidants which play an important role in the prevention of oxidative damage [8]. In the presence of excess oxygen species, inactivation of detoxification systems and degradation of antioxidants, endogenous antioxidative defences are highly effective [3]. In light of the oxidative hypothesis of ischemia-induced cell death, there is an increasing interest focusing on natural products that may ameliorate DND due to cerebral ischemia. Numerous antioxidants and scavengers of ROS have been tested, and many have shown neuroprotective effects [9-13].

Na^+K^+-ATPase is the enzyme, located to the cytoplasmatic membrane responsible for the active transport of sodium and potassium ions in the nervous system, maintaining the ionic gradient necessary for neuronal excitability and regulation of neuronal cell volume. It has been demonstrated that this enzyme is susceptible to

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free radical attack [14,15], and there are some reports showing that Na^+K^+-ATPase activity is decreased after cerebral ischemia [16] and in various chronic neurodegenerative disorders [14].

Resveratrol (RSV: 3,5,4′-trihydroxy-*trans*-stilbene) is found in many plant species such as grapes, berries and peanuts and exhibits pleiotropic health beneficial effects, including anti-oxidant, anti-inflammatory, cardioprotective, neuroprotective and anti-tumor activities [17-21]. Wang and colleagues [22] showed that resveratrol decrease delayed neuronal cell death, reducing the reactive astrogliosis and microglial activation in gerbils. It was demonstrated that resveratrol is the main responsible for cardiovascular benefits associated with moderate wine consumption [23], and this beneficial effect has been attributed to its strong antioxidant activity [17,19]. Although direct neuroprotective effects of resveratrol against oxidative stress have been studied in the PC12 cells and animals [17,24], there is no such investigation in models of global cerebral ischemia in rats. Therefore, considering that an enhancement of antioxidant activities in brain tissue may be potentially beneficial for neuronal recovery from I/R injury, we investigated the effectiveness of resveratrol pretreatment against brain injury caused by transient global cerebral ischemia in rats. In the present study we analyzed cell damage, Na^+K^+-ATPase activity and oxidative stress parameters both in the CA1 subfield of hippocampus and cerebral cortex.

2. Methods

2.1. Experimental procedures

Adult male Wistar rats (290–330 g) were obtained from the Central Animal House of the Department of Biochemistry, Institute for Basic Health Sciences, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals were maintained under a 12/12-h light/dark cycle, in an ambient temperature (24±1°C) colony room. Animals were provided with a constant supply of food (ad libitum) and water. Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Societies for Experimental Biology and experimental protocols were conducted with the approval of the Ethics Committee of the Federal University of Rio Grande do Sul, Brazil.

2.2. Transient global cerebral ischemia

Transient global cerebral ischemia was induced by the four-vessel occlusion method described by [1], with minor modifications [25]. Briefly, rats were anesthetized with a mixture of 70% nitrous oxide, 30% oxygen and 1.5% halothane during surgical operation. Vertebral arteries were permanently electrocoagulated through the alar foramina at the first cervical vertebrae and the common carotid arteries were then exposed, carefully separated from the vagus nerve and isolated. Twenty-four hours later, transient global ischemia was accomplished by bilateral occlusion of the common carotid arteries with aneurysm clips for 10 min. Reperfusion started when aneurysm clips were removed and patency of arteries was confirmed by inspection. The rectal temperature was monitored and maintained at 37±0.5°C with a feedback-controlled heating-pad. Animals which did not lose the righting reflex or convulsed during the ischemic episode were excluded. Sham-operated group underwent the same procedures except for the occlusion of carotids [25].

2.3. Experimental protocols

Resveratrol (courtesy of Pharmascience, Montreal, Canada) was freshly prepared in 50% ethanol [17]. It was administered at the dose of 30 mg/kg, intraperitoneally everyday for 7 days before ischemia, volume not exceeding 0.1 ml/100 g rat weight. The dose of RSV was selected on the basis of earlier reports, which have demonstrated its antioxidant property in different experimental models in doses ranging from 8–40 mg/kg [17,22].

Rats were randomly divided into four groups: Sham + vehicle, Sham + RSV, ischemia + vehicle and ischemia + RSV. After the animals were sacrificed, cortex and hippocampal tissues were isolated for biochemical studies in 1 and 24 h. Another group of animals were prepared for histopathological examination after 7 days of injury. The vehicle-treated group received 0.1 ml of 50% ethanol v/v per 100 g for 7 days and subjected to global cerebral ischemia. The vehicle-treated sham group received 0.1 ml of 50% ethanol v/v /100 g for 7 days. Preliminary experiments confirmed that, in our biochemical and histopathological examinations, the vehicle-treated group was comparable to control.

2.4. Histological analysis

2.4.1. Nissl staining

Neuronal cell loss was assessed by histological examination of Nissl-stained brain sections at the level of the dorsal hippocampus from sacrificed animals 7 days after ischemia. Animals were deeply anesthetized with pentobarbital (50 mg/kg, i.p.) and then transcardially perfused with cold saline followed by 4% paraformaldehyde in phosphate-buffered saline (0.1 M; pH 7.4). Brains were removed and immersed in fixative (4°C overnight). Coronal sections (20 μm) were cut at the level of the dorsal hippocampus (3.3–4.0 mm posterior from bregma) with a cryostat (Leica Microsystems, Germany). Every fourth section was collected and either stained with Cresyl violet or with Fluoro Jade C. The number of surviving pyramidal neurons per 250-μm length of the medial CA1 pyramidal cell layer was counted bilaterally in four sections per animal under a light microscope at 40× magnification. Cell counts from the right and left hippocampus on each of the four sections were averaged to provide a single value (number of neurons per 250 μm length) for each animal.

2.5. Fluoro-Jade C staining

Fluoro-Jade C is a polyanionic fluorescein derivative which sensitively and specifically binds to degenerating neurons. Its high affinity to degenerating neurons with green fluorescence has made it an excellent marker for detecting degenerating neurons. The sections were air dried for 1 h and afterward were immersed in a basic alcohol solution consisting of 1% sodium hydroxide in 80% ethanol for 5 min. They were then rinsed for 2 min in 70% ethanol, following 2 min in distilled water, and then incubated in 0.06% potassium permanganate solution for 10 min. Slides were rinsed with distilled water for 1-2 min and then incubated to a 0.0001% solution of Fluoro-Jade C dissolved in 0.1% acetic acid for 15 min. The slides were then rinsed three times with distilled water for 1 min each. Excess water was drained onto a paper towel and the slides were then air dried. Air-dried slides were cleared in xylene for at least 1 min and a coverslipped was placed with DPX (Sigma) mounting media.

2.6. Measurement of ROS

To assess the ROS formation, 2′,7′-dichlorofluorescin (DCFH-DA, Sigma) was used as a probe [26]. Within the cell, esterases cleave the acetate groups on DCFH-DA, thus trapping the reduced probe (DCFH) intracellularly. ROS in the cells leads to the oxidation of DCFH, yielding the fluorescent product DCF. An aliquot of the sample was incubated with DCFH-DA (100 μM) at 37°C for 30 min, chilling the reaction mixture in ice terminated the reaction. The formation of the oxidized fluorescent derivative (DCF) was monitored at excitation and emission wavelengths of 488 and 525 nm, respectively, using a fluorescence spectrophotometer. The free radical content was quantified using a DCF standard curve and results were expressed as nanomoles of DCF formed per milligram of protein.

2.7. Determination of NO metabolites

Nitrites (NO_2) were determined using the Griess reagent, in which a chromophore with a strong absorbance at 540 nm is formed by reaction of nitrite with a mixture of naphthyl-etilenediamine (0.1%) and sulphanilamide (1%). $NaNO_2$ was used as standard to calculate NO_2 concentrations. Results were expressed as nanomoles per milligram of protein of nitrates plus nitrites [27].

2.8. Na^+,K^+-ATPase activity assay

The reaction mixture for Na^+,K^+-ATPase activity assay contained 5.0 mM $MgCl_2$, 80.0 mM NaCl, 20.0 mM KCl and 40.0 mM Tris–HCl with pH 7.4, in final volume of 200 μl. The reaction was initiated by ATP addition. Controls were carried out under the same conditions with the addition of 1.0 mM ouabain. Na^+,K^+-ATPase activity was calculated by the difference between the two assays as described by Wyse et al. [28]. Released inorganic phosphate (Pi) was measured by the method of Chan et al. [29]. Specific enzyme activity was expressed as nanomoles of Pi released per minute per milligram of protein. All samples were run in duplicate.

2.9. Thiobarbituric acid-reactive substances (TBARS)

TBARS measures malondialdehyde (MDA), a product of lipid peroxidation caused mainly by hydroxyl free radicals. A mixture of 50 μl of 8.1% sodium dodecyl sulfate and 1.5 ml of 20% acetic acid solution was adjusted to a pH of 3.5. Afterward, the mixture was combined with 1.5 ml of 0.8% aqueous solution of thiobarbituric acid and was added to 500 μl of tissue homogenate in a Pyrex tube and heated in a boiling water bath for 60 min [30]. After cooling with tap water, the mixture was centrifuged at 1000×g for 10 min. The organic layer was taken and the resulting pink color was measured in a spectrophotometer at a wavelength of 535 nm. TBARS was calculated as nanomoles of malondialdehyde formed per milligram of protein. The results were reported as nanomoles of TBARS per milligram of protein.

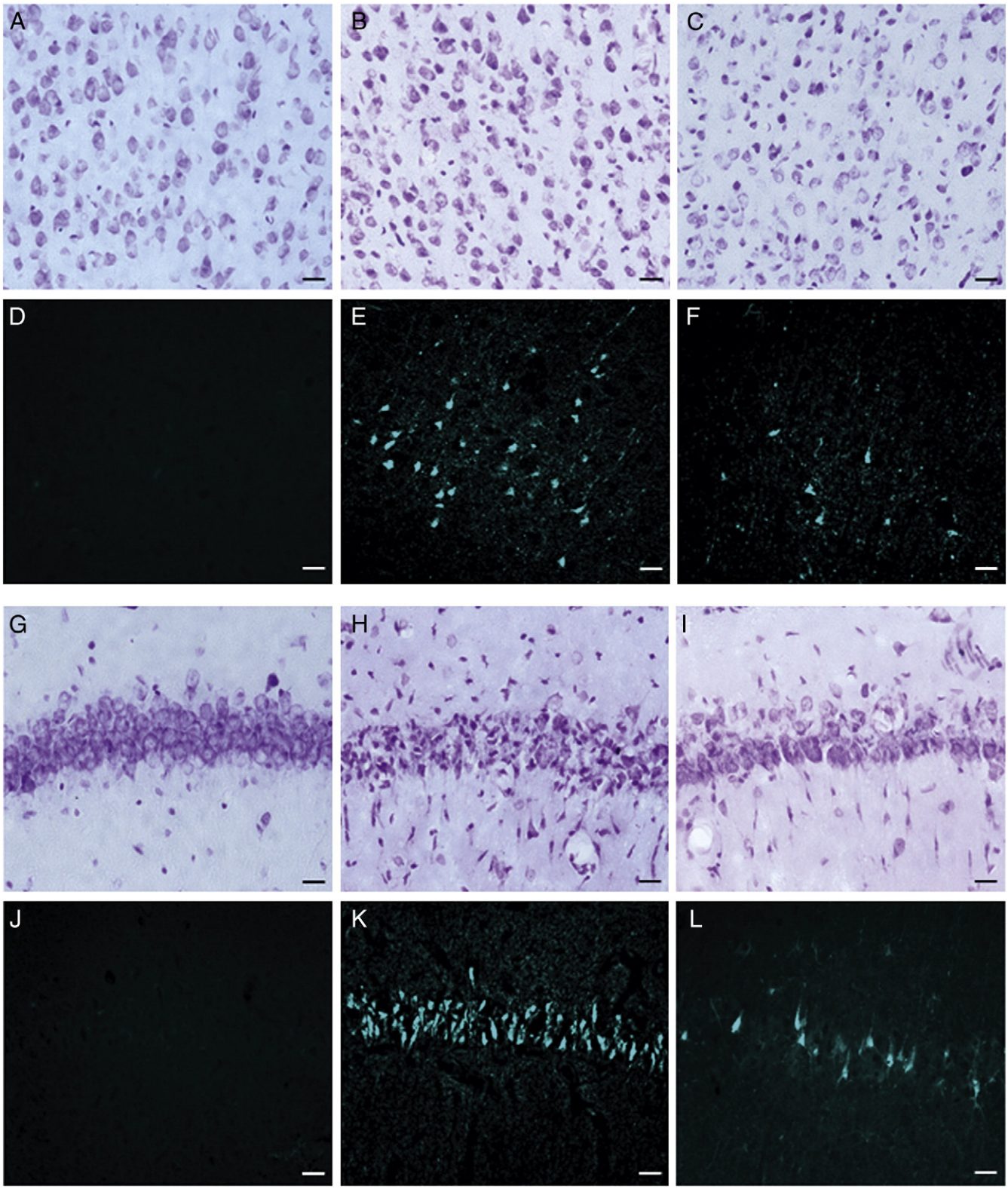

Fig. 1. Representative photomicrographs of Nissl and Fluoro Jade C-stained cells in the cortex and hippocampus (CA1). Vehicle-treated sham group (A and D); vehicle-treated ischemic group (B and E); and RSV-treated ischemic group (C and F) at 7 days of reperfusion in cortex. Vehicle-treated sham group (G and J); vehicle-treated ischemic group (H and K); and RSV-treated ischemic group (I and L) at 7 days of reperfusion in hippocampus. RSV was administered i.p (30 mg/Kg) for 7 days before ischemia (original magnification ×400; scale bar=50μm).

2.10. Total radical-trapping antioxidant potential (TRAP)

TRAP, which represents the quantity of non-enzymatic antioxidant defenses in tissue was determined by measuring the chemiluminescence intensity of luminol induced by 2,2′-azo-bis-(2-amidinopropane) (ABAP) thermolysis [31] in a Wallac 1409 Scintillation Counter. The initial chemiluminescence value was obtained by the addition of 3 ml of 10 mM ABAP, dissolved in 50 mM sodium phosphate buffer pH 7.4, and 10 μl of 5.6 mM luminol into a glass scintillation vial. Ten microliters of 160 μM

Trolox (water-soluble α-tocopherol analogue, used as standard) or tissue supernatant were then added to the vial, producing a decrease in the chemiluminescence value until the antioxidants present were depleted and chemiluminescence reaches its initial values. The time taken by the sample to keep the chemiluminescence low is called induction time and is directly proportional to the antioxidant capacity of the tissue. The induction time of the tissue was compared to that presented by Trolox. Results were reported as nanomoles of Trolox per milligram of protein.

2.11. Antioxidant enzymes

2.11.1. Superoxide dismutase assay

SOD activity is based on the capacity of pyrogallol to autoxidize, a process highly dependent on its substrate, superoxide. [32]. The inhibition of autoxidation of the compound occurs in the presence of SOD, whose activity can be then indirectly assayed spectrophotometrically at 420 nm. A calibration curve was performed with purified SOD as standard, to calculate the activity of SOD present in the samples. The results were reported as units per milligram of protein.

2.11.2. Catalase assay

CAT activity was assayed according to Aebi [33] by measuring the decrease in absorbance at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1% Triton X-100 and 10 mM potassium phosphate buffer, pH 7.0. One CAT unit is defined as 1 μmol of hydrogen peroxide consumed per minute and the specific activity is reported as units per milligram of protein.

2.11.3. Glutathione peroxidase assay

GSH-Px activity was measured according to the method described by Wendel [34] using *tert*-butyl hydroperoxide as substrate. Nicotinamide adenine dinucleotide phosphate (NADPH) disappearance was monitoredspectrophotometrically at 340 nm in a medium containing 2 mM glutathione, 0.15 U/ml glutathione reductase, 0.4 mM azide, 0.5 mM *tert*-butyl hydroperoxide and 0.1 mM NADPH. One GSH-Px unit is defined as 1 μmol of NADPH consumed per minute and the specific activity is represented as units per milligram of protein.

2.12. Statistical analysis

All assays were performed in duplicate and the mean was used for statistical analysis. Data were analyzed by one-way analysis of variance (ANOVA) followed by the Duncan multiple test. All analyses were performed using the SPSS software. Values of $P<.05$ were considered to be significant.

3. Results

3.1. RSV is neuroprotective against I/R-induced DND

Microphotographies of the cortex and hippocampal CA1 subfield for each group are shown in Fig. 1. Histological observation shows that neurons in the cortical layer (Fig. 1A) and CA1 pyramidal cell (Fig. 1G) are clear and moderate-sized with normal ultrastructure in vehicle-treated sham rats. In the vehicle-treated ischemic rats, pyramidal neurons exhibited either significant shrinkage, dark staining appearance with minimal cytoplasm or outright loss of neurons and widespread damage was evident after 7 days (Fig. 1B and H). These neuropathological signs were not present in RSV treated animals, which reduced neuronal loss and moderate morphologic changes (Fig. 1C and I). Fluoro-Jade C histofluorescence stains are remarkably helpful in identifying neurodegeneration, which is indicated by a bright fluorescence. A representative Fluoro-Jade C labeling is presented in the cortex (Fig. 1D–F) and hippocampus (Fig. 1J–L). No Fluoro-Jade C positive fluorescence staining was noted in the cortex (Fig. 1D) and hippocampal (Fig. 1J) regions of sham-operated group. On the other hand, hippocampal and cortex sections from vehicle-treated ischemic rats showed a dramatic increase in Fluoro-Jade C positive cells in the cortical (Fig. 1E) and CA1 pyramidal (Fig. 1K) cell layer, whereas very few positive cells were seen in similar sections of the RSV-treated animals (Fig. 1F and L). Global ischemia induced extensive death of pyramidal cells in the cerebral cortex ($P<.001$ vs. vehicle-treated sham rats) and hippocampal CA1 subfield ($P<.001$ vs. vehicle-treated sham rats) at 7 days post ischemia (Fig. 2). RSV greatly reduced the ischemia-induced neuronal loss in hippocampus ($P<.01$ vs. vehicle-treated ischemia and vehicle-treated sham rats) and cerebral cortex ($P<.01$ vs. vehicle-treated ischemia and vehicle-treated sham rats), as shown in Fig. 2.

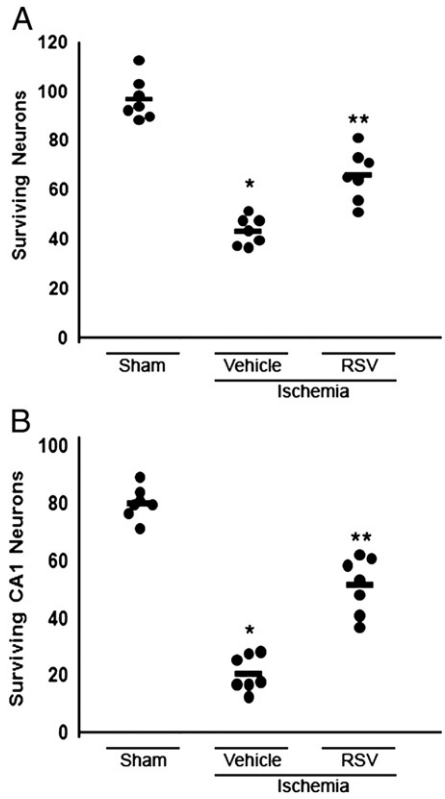

Fig. 2. Influence of RSV on numbers of survival neurons in cortex (A) and hippocampus (CA1) (B) in rats after 7 days of reperfusion. Data are mean±S.D. ($n=7$ each). One-way ANOVA revealed significant interactions between ischemia and RSV. *$P<.001$ compared with vehicle-treated sham group; **$P<.01$ compared with vehicle-treated ischemic and vehicle-treated sham group.

3.2. Biochemical analysis

3.2.1. ROS and NO content

The fluorogenic compound DCFH-DA is one of the most prominent markers to reflect the overall oxidative status in cells. As shown in Fig. 3A–B, rats that had undergone transient global cerebral ischemia for 10 min followed by 1 h of reperfusion exhibited 100% and 99% increase in ROS production in the cortex ($P<.01$) and hippocampus ($P<.01$), respectively, as compared with vehicle-treated sham animals. Decreases in basal levels were recorded at 24 h of reperfusion in cortex and hippocampus. The I/R induced ROS overproduction at 1 h of reperfusion was significantly decreased by RSV in cortex ($P<.01$) and hippocampus ($P<.01$) (Fig. 3A-B). No alterations in DCFH levels were observed in sham animals treated with or without RSV.

Nitrite is the major end-product of nitric oxide, which was undertaken to monitor NO production during ischemia. NO content significantly increased 1 h after transient global cerebral ischemia in cortex ($P<.01$) and hippocampus ($P<.001$) when compared to vehicle-treated sham rats (Fig. 3C-D). After 24 h of cerebral injury, NO content returned to basal level. Administration of RSV significantly inhibited the increase of NO content in cortex ($P<.05$) and hippocampus ($P<.01$) when compared to vehicle-ischemic group (Fig. C-D). No alterations in NO content was observed in sham animals, treated with or without RSV.

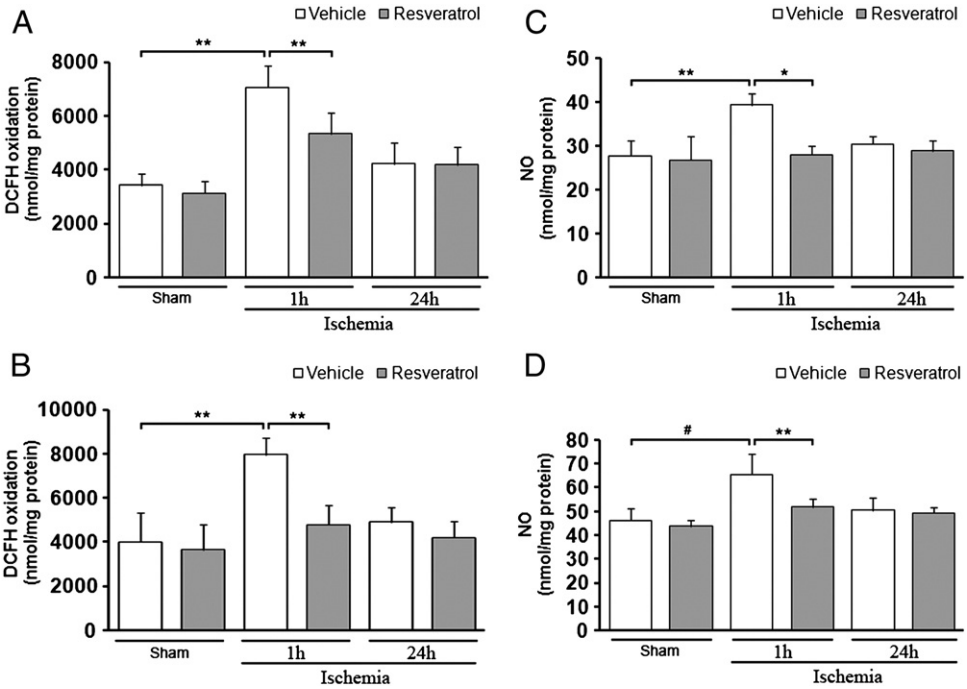

Fig. 3. Effect of RSV on ROS generation (A and B) and NO content (C and D) from cortex (A and C) and hippocampus (B and D) of rats at 1h and 24h after global cerebral ischemia. ROS levels were assessed by dichloroflurescein (DCF) assay. NO content was assessed by Griess reagent. Data are mean±S.D. (n=6 each). *P<.05; **P<.01; #P<.001.

3.2.2. Lipid peroxidation

When subjected to global cerebral ischemia, animals showed a significant increase in MDA in the cortex (P<.001) and hippocampus (P<.05) after 1 h (Fig. 4A–B). After 24 h of cerebral injury, MDA levels remained increased in the cortex (P<.01) and hippocampus (P<.05). As shown in Fig. 4A, ischemia-mediated lipid peroxidation decreased significantly in RSV-treated rats in cortex 1 (P<.05) and 24 h (P<.05) after ischemia. Hippocampus showed the same pattern and reversed the alterations in MDA levels in 1 (P<.05) and 24 h (P<.05) after ischemia (Fig. 4B). No alterations in MDA levels were observed in sham operated animals treated with or without RSV.

3.2.3. Na^+K^+-ATPase

As shown in Fig. 5(A–B), ischemia significantly reduces Na^+,K^+-ATPase activity in the cortex (P<.01) and hippocampus (P<.05) after 1 h when compared to vehicle-treated sham groups. After 24 h of reperfusion, maximal decrease of Na^+,K^+-ATPase activity was observed in the cortex (P<.01) and hippocampus (P<.05). In RSV-treated ischemia, the decrease in Na^+,K^+-ATPase was significantly smaller in the cortex (1 h, P<.05 and 24 h, P<.05) (Fig. 5A) as well as in the hippocampus (1 h, P<.05 and 24 h, P<.05) after ischemia (Fig. 5B). The administration of RSV and vehicle in the sham animals did not alter Na^+,K^+-ATPase activity.

3.2.4. The activities of antioxidant enzymes

The effect of RSV on the antioxidant capacity of hippocampus and cortex homogenates was studied by determining TRAP, which represents non-enzymatic antioxidant quantity. It can be observed in Fig. 5C that global cerebral ischemia decreased TRAP in the cortex in 1 h (P<.05) and 24 h (P<.05) when compared to vehicle-treated sham group. Furthermore, ischemia significantly decreased TRAP in the hippocampus in 1 h (P<.05) and 24 h (P<.05) when compared to vehicle-treated sham groups (Fig. 5D). RSV-treated rats significantly prevented the alterations on the antioxidant capacity in the cortex 24 h (P<.05) after reperfusion when compared to vehicle-treated ischemic group (Fig. 5C). Antioxidant capacity in hippocampus was significantly prevented by RSV-treatment in 1 h (P<.05) and 24 h (P<.05) after reperfusion when compared to ischemia-group (Fig. 5D).

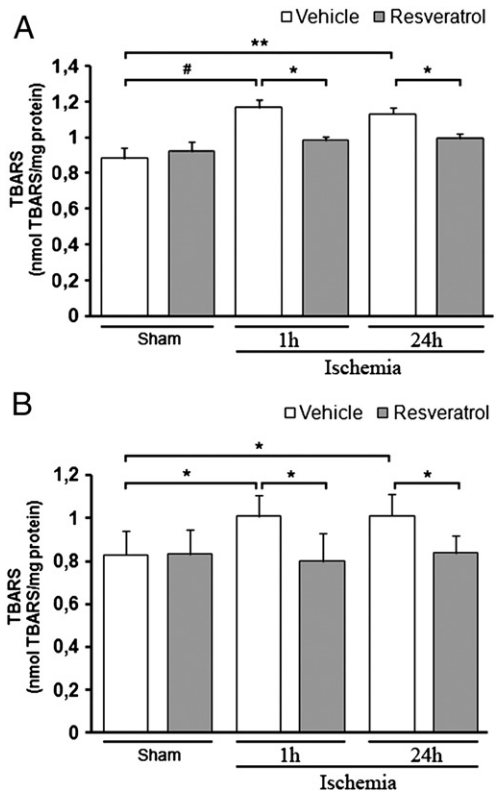

Fig. 4. Effect of RSV on lipid peroxidation level in rats cortex (A) and hippocampus (B) at 1 and 24 h following 10 min of global cerebral ischemia. TBARS is expressed as nanomoles of malondialdehyde per mg protein. Data are mean±S.D. (n=6 each). *P<.05; **P<.01; #P<.001.

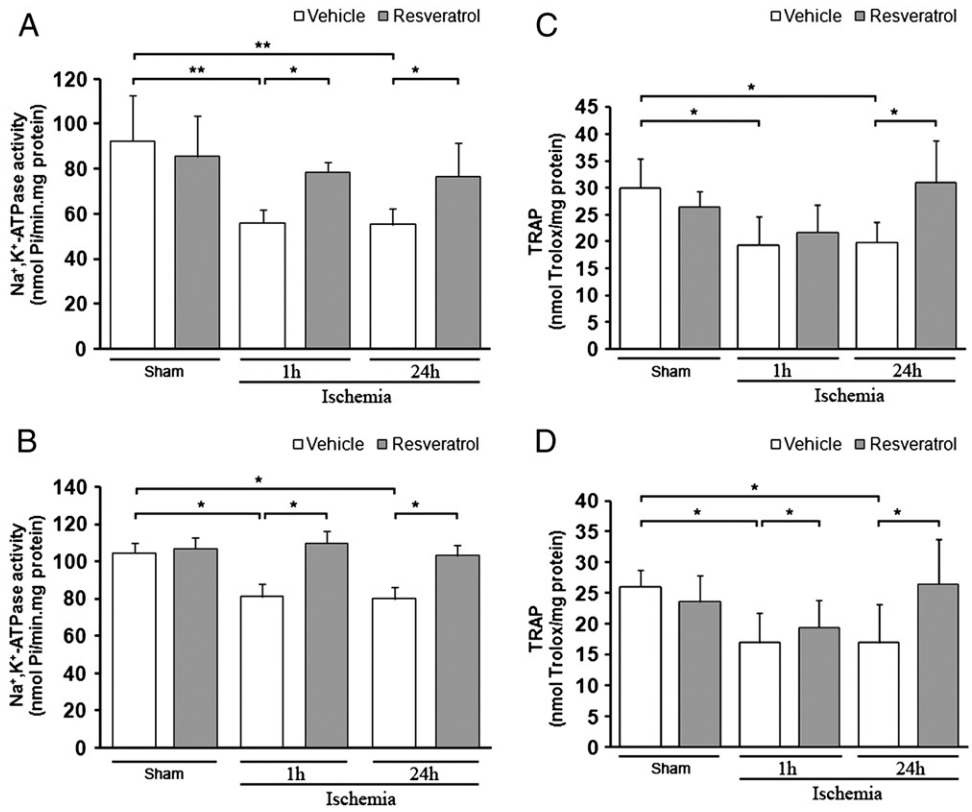

Fig. 5. Effect of global cerebral ischemia after 1 and 24 h and RSV treatment on Na^+,K^+-ATPase activity and antioxidant capacity (TRAP) in cortex (A and C) and hippocampus (B and D). Na^+,K^+-ATPase is expressed in nanomoles of Pi per milligram of protein and TRAP in nanomoles of Trolox per milligram of protein. Data are mean±S.D. (n=6 each). *P<.05; **P<.01.

After 1 h and 24 h of reperfusion, the hippocampus of ischemic animals showed a significant increase in SOD and GPx, whereas there was a significant decrease in catalase, as compared with the vehicle-treated sham animals (Table 1). Cortex showed a significant increase in SOD and a significant decrease in catalase, as compared with the vehicle-treated sham animals (Table 1). Treatment with RSV markedly reversed the alterations in enzymatic parameters brought about by I/R. The values were almost restored to near-normal levels with no significant differences versus the vehicle-treated sham group.

Table 1
Effect of RSV on hippocampus and cortex oxidative stress parameters (SOD, catalase and GPx levels) after global cerebral ischemia

Groups (n=6)	Time of reperfusion (h)	SOD (U/mg protein)	CAT (U/mg protein)	GPx (U/mg protein)
Hippocampus				
Control	–	6.57±0.21	4.21±0.19	21.03±2.20
Control+RSV	–	7.46±0.71	4.02±0.32	23.31±2.85
Isch.+vehicle	1	7.78±0.74a*	2.98±0.41a#	25.91±0.59a#
Isch.+RSV	1	6.41±0.25b#	4.16±0.51b#	22.71±1.01b*
Isch.+vehicle	24	7.93±1.15a*	3.26±0.45a*	26.01±3.04a#
Isch.+RSV	24	6.36±1.04c#	4.24±0.92c*	21.50±2.71c#
Cortex				
Control	–	4.69±0.18	4.68±0.63	23.74±2.73
Control+RSV	–	4.94±0.19	4.47±0.69	26.11±2.97
Isch.+vehicle	1	5.29±0.38a*	3.00±0.8a*	24.48±1.74
Isch.+RSV	1	4.97±0.32b*	4.48±1.03b*	21.97±1.65
Isch.+vehicle	24	5.26±0.29a*	3.10±0.91a*	24.11±0.81
Isch.+RSV	24	4.76±0.36c*	4.41±1.20c*	21.93±2.68

RSV (30 mg/kg) was administered intraperitoneally 7 days before occlusion. Values are expressed as mean±S.D. with n=6 per group.
*P<.05 and #P<.01: a, vs. control animals; b, vs. ischemic animals 1 h after reperfusion and c, vs. ischemic animals 24 h after reperfusion (tested using one-way ANOVA followed by Duncan multiple comparison test).

4. Discussion

The removal of pathologically generated free radicals during I/R seems to be a viable approach to neuroprotection. The present study demonstrates that pretreatment with RSV prevented both structural and biochemical abnormalities in rats subjected to global cerebral ischemia. RSV pretreatment protected the brain tissue against the increase of lipid peroxidation, NO levels and ROS formation as well as the decrease of Na^+K^+-ATPase activity and disrupted antioxidant defenses (enzymatic and non-enzymatic). As oxidative stress is closely associated with ischemic neuronal death after global cerebral ischemia, the preservation of the neuronal integrity in our model was provided presumably via the direct and indirect antioxidant properties of RSV [17,18]. Supporting this assumption, RSV prevented the loss of pyramidal cells in the hippocampal CA1 layer and cerebral cortex, a finding consistent with a previously published studies reporting that RSV ameliorates neuronal damage in CA1 following I/R [22,35].

Our results have shown a sustained increase of lipid peroxidation markers following transient global ischemia, which was at 1 h and 24 h of recirculation. These findings are consistent with other studies, which have found that the early increased level of lipid peroxidation persists for several days (1 h–4 days) after brief global cerebral ischemia in the hippocampus and 1 h to 24 h in cortex [4,36]. ROS production in the cortex and hippocampus returned to basal levels after being increased at 1 h and at 24 h after transient global cerebral

ischemia. These results are in agreement with other studies, which have found an early increase in ROS generation and a decrease in the hippocampus 24 h after brief global cerebral ischemia. In addition, high levels of LPO were observed in the hippocampus and cortex 24 h after ischemia. This may possibly involve mechanisms that account for the late increase in oxidative damage, delaying the induction of ROS-generating enzymes like cyclooxygenase-2 [37] and nitric oxide synthase [38]. RSV pretreatment decreased LPO levels, an effect that could be attributed to its capacity to scavenge ROS, indicating that the neuroprotection conferred by RSV is due to its anti-oxidative effect of attenuating ROS formation and lipid peroxidation. RSV was showed to restore decrease lipid peroxidation in different tissues exposed to ischemia [17,39,40]. A number of studies have demonstrated the antioxidant properties of RSV, for example, its ability to protect against oxidative DNA damage in stroke-prone hypertensive rats [41], to suppress lipid peroxidation [42] and to inhibit cerebral mitochondrial ROS production [43].

One of the ROS that elevates in cerebral ischemia is NO nevertheless; its precise role in this neuropathology remains controversial [7,44]. NO is beneficial as a messenger or modulator, but in conditions such as oxidative stress, it is potentially toxic. The toxic effects of NO may be attributed to peroxynitrite ($ONOO^{\cdot -}$), which is a reaction product of NO with superoxide ($O_2^{\cdot -}$) [45]. It may be suggested that diminishing the formation of $ONOO^{\cdot -}$ might reduce the potential for damage to neurons and supporting elements in the brain observed with ischemia and reperfusion. NO synthase activity and NO release are greatly increased in the acutely ischemic brain [46]. We showed that measurement of NO in the hippocampus and cortex homogenate revealed that all were significantly increased response to I/R. The data is consistent with increased oxidative stress. The level of NO in the brain was diminished in response to treatment with RSV. RSV was shown to increase NO levels up to basal levels in normal conditions [47-49]. Under physiological conditions, NO is an important endogenous vasodilator that regulates cerebral blood flow, and mediate vascular response to a diverse group of stimulations. Recently, Kiziltepe et al., [39] showed that RSV pretreatment increased NO levels in plasma during ischemia but not during the reperfusion period. Therefore, we suggest that our findings are consistent with this report and decreasing NO in the reperfusion period could reduce the delayed neuronal death induced by oxidative stress.

Neuronal damage caused by global cerebral ischemia is associated with an imbalance in ionic homeostasis. It has been shown that inhibition of Na^+,K^+-ATPase activity by ouabain induces the release of neurotransmitters including glutamate, which is proposed to play a major role in neuronal death after excitotoxic and ischemic insults [39]. A possible mechanism to explain ischemia-induced inhibition of Na^+,K^+-ATPase is the well-documented increase in free fatty acids and ROS following ischemia [50]. It has been demonstrated that the structural properties [51] and lipid composition [52] of synaptosomal membrane are essential for enzyme activity and that even low concentrations of free fatty acids and free radicals inhibit Na^+,K^+-ATPase activity of rat brain. Therefore, the period of reperfusion is highly suitable for lipid peroxidation. Previous findings suggest that reperfusion produces a decrease of Na^+,K^+-ATPase activity in early periods that is maintained during 24 h, corroborating findings from Wyse and colleagues [16]. The present data shows that RSV pretreatment prevents the inhibition of Na^+,K^+-ATPase activity in the hippocampus and cortex, suggesting that during cerebral ischemia, RSV decreases ROS formation and impairment in membrane permeability due to lipid peroxidation causing a decrease in Na^+,K^+-ATPase enzyme activity. In regards to the possible consequences of the inhibition of Na^+,K^+-ATPase activity to neural cellular metabolism and function, it should be stressed that there is increasing evidence suggesting that alterations in Na^+,K^+-ATPase activity may be a link between many common neurotoxic mechanisms in neurons [14]. In this context, blockage of the Na^+,K^+-ATPase activity secondary to energy depletion and free radical attack may be a common event in the apoptotic cascade [53].

Oxidative stress is the result of a redox imbalance between the generation of ROS and the compensatory response from the endogenous antioxidant network. In the present study, SOD and GPx activities increased in ischemic rats. The increase in GPx activity may be a compensatory response for an increase in endogenous H_2O_2 production in ischemic brain because ROS formation promotes the oxidation of fatty acids with resulting H_2O_2 formation [54]. The increase in SOD activity could be due to its induction by increased production of superoxide and H_2O_2 was reported to act as an inducer of tissue SOD. Increases in both SOD and GPx activities may thus be an adaptive response for increased oxidative stress in the brain tissue. RSV, by scavenging ROS, prevents the elevation of those antioxidant enzyme activities in ischemic rat brain. The presence of molecules and macromolecules capable of transforming active radicals into inactive species (e.g., chain-breaking antioxidants) constitutes one of these defenses. This type of defense includes a variety of compounds bearing different reactive centers (e.g., phenols, thiols) with widely different hydrophobicities that allow the trapping of both hydrophobic and hydrophilic radicals. In this regard, there is great interest in the evaluation of the total reactive antioxidant potential (TRAP), and several procedures have been developed to measure it in biological fluids and animals [55]. The interest in this type of determination resides in that they can provide information regarding the system's capacity to withstand oxidative stress disbalances. In this view, decreasing TRAP to energy depletion and free radical attack may be an event in the global cerebral ischemia-induced delayed neuronal death. RSV pretreatment brought TRAP measurements back to normal levels in the cortex and hippocampus and contributed to the neuroprotective effect after transient global cerebral ischemia.

In conclusion, this study suggests that RSV pretreatment attenuates ischemia/reperfusion-induced cerebral injury decreasing oxidative stress and maintaining Na^+K^+-ATPase activity. These observations suggest that RSV may be a clinically viable protective agent against a variety of conditions where cellular damage is a consequence of oxidative stress. In addition, RSV may have the potential to be used in the prevention of neurodegenerative diseases such as forebrain ischemia.

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