

Protective Effects of Ascorbic Acid on Behavior and Oxidative Status of Restraint-Stressed Mice

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Abstract Studies have demonstrated an association between stressful conditions and the onset of clinical depression. Considering the antidepressant-like properties of ascorbic acid in both experimental and clinical approaches, we evaluated the beneficial effect of this vitamin on restraint stress-induced behavioral and neurochemical alterations. Acute restraint stress caused a depressive-like behavior in the forced swimming test, accompanied by increased lipid peroxidation (cerebral cortex and hippocampus); increased superoxide dismutase (cerebral cortex and hippocampus), glutathione reductase (cerebral cortex), and glutathione peroxidase (cerebral cortex and hippocampus) activities; and elevated expression of Bcl-2 (hippocampus). Oral administration of ascorbic acid (1 mg/kg) or fluoxetine (10 mg/kg) 1 h before restraint stress prevented the stress-induced increase on immobility time in the forced swimming test. Moreover, this vitamin reduced lipid peroxidation to control levels and restored the activity of superoxide dismutase, glutathione reductase, and glutathione peroxidase. Ascorbic acid had no effect on the increased level of Bcl-2 induced by stress. Glutathione levels, glycogen synthase kinase-3 β phosphorylation, and Bax expression were not altered by stress or ascorbic acid administration. Besides reinforcing the antioxidant potential of ascorbic acid, our results support the notion that oxidative stress plays a role in the pathogenesis and treatment of stress-induced depression.

Keywords Ascorbic acid · Acute restraint stress · Forced swimming test · Oxidative stress · Antioxidant

Introduction

Stress has been reported as a predisposing and precipitating factor of depression, particularly in genetically predisposed individuals (Checkley 1996). The mechanisms underlying the basis of stress-related depression still remain elusive despite much effort being pursued in this direction. Although it is impossible to recapitulate all aspects of a complex human disease such as depression in animals, a number of experimenter-applied animal models have been developed in an attempt to determine the influence of stress on the development of depression, of which, restraint stress is one of the most widely means of stressing animals, fundamentally because it is straightforward and painless and without lasting debilitation (Buynitsky and Mostofsky 2009; O'Mahony et al. 2010; Shoji and Mizoguchi 2010).

Stress is one of the most important contributory factors in the stimulation of intracellular pathways leading to the increased free radical generation. Numerous reports have revealed that restraint stress can affect central nervous system (CNS) functions by producing neurochemical and hormonal abnormalities associated with imbalance of antioxidant status. The oxidative stress resulting from increased intracellular reactive oxygen species (ROS), such as superoxide, hydrogen peroxide, and hydroxyl radicals, disturbs homeostasis within the neurons and can lead to cell death (Kovacheva-Ivanova et al. 1994; Oishi et al. 1999; Radak et al. 2001). Many studies have shown that restraint stress induces increased lipid peroxidation (Atif et al. 2008; Ahmad et al. 2012; García-Fernández et al. 2012) and increases or decreases antioxidant enzyme

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activities in different brain regions of rodents, depending on the severity and duration of restraint stress protocol (Fontella et al. 2005; Pajović et al. 2006; Kumari et al. 2007; Atif et al. 2008; García-Fernández et al. 2012).

Of note, the execution of the apoptotic machinery is also a function of the balance between the pro-apoptotic and the anti-apoptotic members of the Bcl-2 family (such as Bax/Bcl-2), with the fate of the cell being determined by the tilt in the ratio towards one or the other (Adams and Cory 2007). Several lines of evidence suggest that one of the mechanisms through which Bcl-2 family proteins protect against cell death involves enhanced antioxidant defenses in these cells (Kowaltowski and Fiskum 2005).

Another component that has been implicated in the pathophysiology of depression is the enzyme glycogen synthase kinase-3 β (GSK-3 β), a multi-tasking serine/threonine kinase which regulates many aspects of neuronal function, such as gene expression, neurogenesis, synaptic plasticity, neuronal structure, and neuronal death and survival (Frame and Cohen 2001; Doble and Woodgett 2003; Jope and Johnson 2004; Beaulieu et al. 2009; Li and Jope 2010). Behavioral studies in rodents, including investigations of the mechanisms of action of therapeutic agents, have provided substantial evidence of the association between GSK-3 β and depressive disorder (Gould et al. 2004; Li et al. 2004; Beaulieu et al. 2008). These studies propose that increased GSK-3 β activity in specific cellular locations, pathways, and circuits promotes vulnerability to mood disorders and that inhibition of GSK-3 β is an important component of the therapeutic actions of antidepressants.

In spite of the wide-ranging, continuously expanding arsenal of antidepressants and intensive research on depression, available antidepressants have serious limitations and the management of severe, recurrent mood disorders as well as antidepressant-resistant refractory mood disturbances has not yet completely been solved (Trivedi et al. 2006). In an attempt to identify new pharmacological agents to improve therapeutic armamentarium for depression, the antidepressant-like potential of ascorbic acid, a water-soluble vitamin with neuroprotective and antioxidant properties (Rice 2000), has been investigated by our group. Indeed, clinical studies reported that the administration of ascorbic acid relieved adrenocorticotrophic hormone-induced depression in a child (Cocchi et al. 1980) and decreased scores in a Beck Depression Inventory in healthy young adults (Brody 2002), indicating mood improvement. In mice, we showed that the administration of ascorbic acid produces an antidepressant-like effect in the tail suspension test (TST) by a mechanism that is dependent on the interaction of this vitamin with the monoaminergic systems (Binfaré et al. 2009), *N*-methyl-D-aspartate (NMDA) receptors and the L-arginine–nitric oxide (NO)–cyclic guanosine monophosphate pathway (Moretti et al. 2011) and potassium channels (Moretti et al. 2012a).

Further supporting the idea that ascorbic acid has antidepressant potential, it was shown that this vitamin is able to reverse the chronic unpredictable stress-induced depressive-related behaviors (Moretti et al. 2012b).

Considering this background, in the present study we have made an attempt to explore the neuroprotective effect of ascorbic acid against the restraint stress-induced depressive like-behavior, alterations in parameters of oxidative stress, and GSK-3 β phosphorylation and Bcl-2 and Bax expression in the cerebral cortex and hippocampus of mice.

Materials and Methods

Animals

The behavioral experiments were conducted using female Swiss mice (35–45 g), maintained at 20–22 °C with free access to water and food, under a 12:12-h light/dark cycle, with lights on at 7:00 a.m. The animals were caged in groups of 15 in a 41×34×16-cm cage. All behavioral tests were carried out between 9:00 a.m. and 6:00 p.m. The animals were used according to the NIH Guide for the Care and Use of Laboratory Animals, and the experiments were performed after approval of the protocol by the Ethics Committee of the Institution. All efforts were made to minimize animal suffering and to reduce the number of animals used in the experiments.

Drugs and Treatment

Ascorbic acid and fluoxetine (obtained from Sigma Chemical Co., St. Louis, USA) were dissolved in distilled water and administered orally (p.o.) at a dose of 1 and 10 mg/kg, respectively, 1 h before the acute restraint stress procedure. Ascorbic acid solution was freshly prepared before administration and administered in a volume of 1 ml/kg. To develop this study, mice were divided into four groups as follows: (1) nonstressed + vehicle; (2) nonstressed + ascorbic acid; (3) stressed + vehicle; and (4) stressed + ascorbic acid. Fluoxetine was added as a positive control in the forced swimming test (FST) and open field test in another set of experiments.

Restraint Stress Procedure

Restraint stress protocol was adapted from a previously described procedure (Poleszak et al. 2006; Kumar and Goyal 2008; Zafir et al. 2009). The animals were divided into four groups as mentioned above. Stressed groups were administered with vehicle or ascorbic acid, and 1 h after the treatment, they were submitted to the stress protocol. The immobilization was applied for a period of 7 h using an individual rodent restraint device made of Plexiglas fenestrate.

This restrained all physical movement without submitting the animal to pain. The animals were deprived of food and water during the entire period of exposure to stress. After 7 h, the animals were released from their enclosure, and 40 min post-release, the animals were submitted to behavioral tests or biochemical evaluations. Nonstressed groups were treated with vehicle or ascorbic acid and were kept without food and water during the entire period of stress.

Behavioral Tests

Forced Swimming Test

Briefly, mice were individually forced to swim in an open cylindrical container (diameter 10 cm, height 25 cm), containing 19 cm of water (depth) at 25 ± 1 °C; the duration of immobility was measured during the last 4 min of the 6-min test period (Porsolt et al. 1977) by observers blind to the treatment conditions. Each mouse was judged to be immobile when it ceased struggling and remained floating motionless in the water, making only those movements necessary to keep its head above water. A decrease in the duration of immobility is indicative of an antidepressant-like effect (Porsolt et al. 1977), whereas an increase of immobility time, when compared with the control group, is associated with depressive-like effect (Brocardo et al. 2007).

Open Field Test

The locomotor activity, assessed in an independent group of animals, was performed in an open field test as described previously (Moretti et al. 2011; Budni et al. 2007). The apparatus consisted of a wooden box measuring $40 \times 60 \times 50$ cm high. The floor of the arena was divided into 12 equal squares. The number of squares crossed with all paws (crossings) was manually counted in a 6-min session. The light was maintained at minimum to avoid anxiety behavior. The apparatus was cleaned with a solution of 10 % ethanol between tests in order to hide animal clues.

Biochemical Analysis

Tissue Preparation

For evaluation of activity of antioxidant enzymes, glutathione (GSH) levels, and thiobarbituric acid reactive substances (TBARS) formation, mice were killed by decapitation and the cerebral cortices and hippocampi were removed and homogenized (1:10 w/v) in HEPES buffer (20 mM, pH 7.0). The tissue homogenates were centrifuged at $16,000 \times g$, at 4 °C for 20 min, and the supernatants obtained were used for the determination of enzymatic activities and for the

quantification of GSH and TBARS levels. The protein content was quantified by the method of Lowry et al. (1951), using bovine serum albumin as standard.

For Western blotting analysis, cerebral cortices and hippocampi were dissected, mechanically homogenized in 500 ml of sample buffer (200 mM Tris, 40 mM EDTA, 4 % SDS, pH 6.8), and immediately boiled for 5 min. Sample dilution solution (1:4 vol/vol; 40 % glycerol, 50 mM Tris and minimal bromophenol blue) and β -mercaptoethanol were added to each sample for a final concentration of 5 %. Protein content was estimated at 620 nm wavelength and the concentration calculated using a standard curve with bovine serum albumin (Peterson 1977).

Activity of Antioxidant Enzymes

Hippocampal and cerebrocortical glutathione reductase (GR) activity was determined based on the protocol developed by Carlberg and Mannervik (1985). Briefly, GR reduces glutathione disulfide (GSSG) to GSH at the expense of NADPH, whose disappearance was followed at 340 nm. Hippocampal and cerebrocortical glutathione peroxidase (GPx) activity was determined based on the protocol developed by Wendel (1981) by indirectly measuring the consumption of NADPH at 340 nm. The GPx uses GSH to reduce the *tert*-butyl hydroperoxide, producing GSSG, which is readily reduced to GSH by GR using NADPH as a reducing equivalent donor. Superoxide dismutase (SOD) activity was assayed spectrophotometrically as described previously (Misra and Fridovich 1972). This method is based on the capacity of SOD to inhibit autoxidation of adrenaline to adrenochrome. The color reaction was measured at 480 nm. One unit of enzyme was defined as the amount of enzyme required to inhibit the rate of epinephrine autoxidation by 50 %. The enzymatic activity was expressed as units (U) per milligram protein.

GSH Levels

Hippocampal and cerebrocortical GSH levels were measured as nonprotein thiols based on the protocol developed by Ellman (1959). Hippocampal and cerebrocortical homogenates were precipitated in cooled trichloroacetic acid 10 % and centrifuged at $15,000 \times g$ for 2 min, and the supernatant was incubated with DTNB in a 1-M phosphate buffer, pH 7.0. Absorbances were measured at 412 nm. A standard curve of reduced glutathione was used to calculate GSH levels.

TBARS Formation

TBARS were determined in the hippocampal and cerebrocortical homogenates using the method described by Ohkawa et

al. (1979), in which malondialdehyde (MDA), an end-product of lipid peroxidation, reacts with thiobarbituric acid to form a colored complex. The samples were incubated at 100 °C for 60 min in an acid medium containing 0.45 % sodium dodecyl sulfate and 0.67 % thiobarbituric acid. After centrifugation, the reaction product was determined at 532 nm using MDA as standard.

Western Blotting

The samples (60 mg of total protein/track) were separated by SDS-PAGE using 12 % gels, and the proteins were transferred to nitrocellulose membranes using a tank transfer system at 100 V and 270 mA for 1 h (Mini-PROTEAN Tetra Cell Electrophoresis System, Bio-Rad, Hercules, CA, USA) (Cordova et al. 2004). The membranes were blocked (1 h) with 5 % skim milk in TBS (10 mM Tris, 150 mM NaCl, pH 7.5). p-GSK-3 β total and phosphorylated forms and Bax and Bcl-2 expression were detected using specific antibodies incubated overnight diluted in TBS-T (10 mM Tris, 150 mM NaCl, 0.1 % Tween-20, pH 7.5) containing 2.5 % BSA in the dilutions 1:1,000. Next, the membranes were incubated with anti-rabbit peroxidase-linked secondary antibody (1:5,000) for 1 h and the reactions developed by chemiluminescence (LumiGLOH, Cell Signaling, Beverly, MA, USA). All blocking and incubation steps were followed by three washes (5 min) of the membranes with TBS-T. The optical density (OD) of the bands was quantified using ScionImageTM (Frederick, MD, USA). The phosphorylation levels of GSK-3 β were determined as a ratio of OD of the phosphorylated band over OD of the total band. The expression of Bcl-2 and Bax were determined as a ratio of OD of the Bcl-2 or Bax band over OD of the β -actin band. Data were expressed as percentage of the control (considered as 100 %).

Statistical Analysis

All data are presented as mean \pm SEM. Differences among experimental groups were determined by two-way ANOVA followed by Duncan's multiple range post hoc test. A value of $p < 0.05$ was considered to be significant.

Results

Behavioral Observations

Immobility Time in the Forced Swimming Test

The most widely used behavioral assay for detecting potential antidepressant-like activity is the FST, which was originally developed by Porsolt et al. (1977). This test has been

shown to have high predictive validity for antidepressant activity. To evaluate the influence of treatment of mice with ascorbic acid or fluoxetine on the depressive-like behavior elicited by acute restraint stress procedure, the immobility time of mice in the FST was measured (Fig. 1a, c). The two-way ANOVA revealed significant differences for stress procedure \times ascorbic acid treatment interaction [$F(1, 25)=5.150$, $p < 0.05$] and for stress procedure \times fluoxetine treatment interaction [$F(1, 27)=6.439$, $p < 0.05$]. Post hoc analyses indicated that restraint stress for 7 h significantly increased the immobility time in the FST as compared to control mice ($p < 0.01$). Ascorbic acid (Fig. 1a) or fluoxetine (Fig. 1c) administration prevented the depressive-like behavior induced by stress ($p < 0.01$). In nonstressed mice, ascorbic acid or fluoxetine administration did not modify the immobility time in the FST in comparison with the control group.

Locomotor Activity

To rule out nonspecific motor effects of stress or treatments that could influence activity in the FST, the open field test was used for assessing locomotor activity in our experimental groups after 7 h of physical restraint. No alteration in spontaneous locomotion was observed, as shown in Fig. 1b, d.

Biochemical Observations

Lipid Peroxidation

Because oxidative stress and lipid oxidative damage have been reported as important outcomes in depressive-like disorders (Bilici et al. 2001), MDA (measured as TBARS), a late product of lipid peroxidation, was measured in the hippocampus and cerebral cortex of animals. The presence of MDA is indicative of prooxidative damage to the membranes, compromising their functions and overall cellular integrity (Hernández-Martínez et al. 2011). As shown in Fig. 2, TBARS levels were significantly increased in the cerebral cortex ($p < 0.01$) and hippocampus ($p < 0.05$) of stressed mice as compared to nonstressed mice. Treatment with ascorbic acid produced a significant reduction in TBARS levels in both encephalic structures of stressed mice ($p < 0.01$). Ascorbic acid administration to nonstressed mice did not produce any significant effect on TBARS levels.

Antioxidant Profile

GSH is the main nonprotein thiol of the mammalian cell. As an important antioxidant, GSH plays a role in the detoxification of a variety of electrophilic compounds and peroxides. In view of its essential function in the maintenance of cellular redox, we evaluated the levels of GSH in our

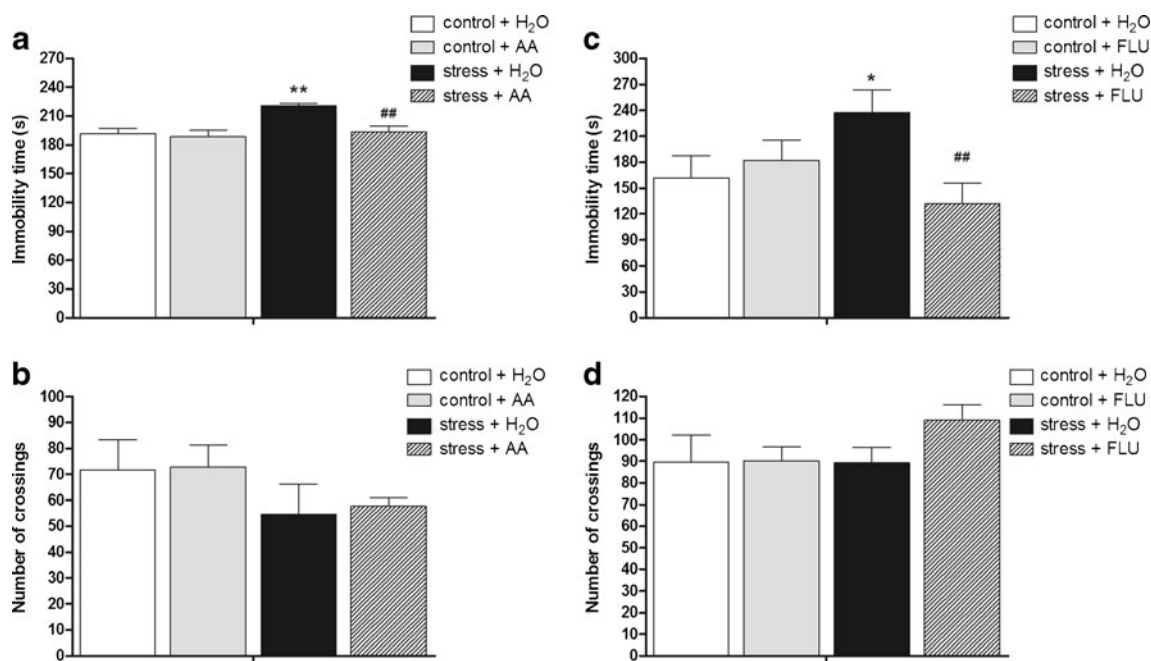


Fig. 1 Effect of treatment with ascorbic acid (1 mg/kg) or fluoxetine (10 mg/kg) on immobility time in the FST (a, c) and on locomotor activity (b, d) in mice submitted to acute restraint stress procedure.

Bars represent means \pm SEM of seven to eight mice. * p <0.05, ** p <0.01 vs. control mice, ## p <0.01 vs. stress + vehicle group

experimental protocol. As can be observed in Fig. 3a, no significant statistical alterations were observed in GSH levels in the cerebral cortex and hippocampus of stressed mice compared to nonstressed animals, both injected with vehicle. Also, treatment with ascorbic acid did not alter GSH levels in the evaluated structures, independent on the stress condition.

Because of its involvement in the pathophysiology of depression and its primary role in the mechanism of dismutation of superoxide to H₂O₂, SOD activity was measured in our study. As depicted in Fig. 3b, SOD activity was significantly increased in the cerebral cortex (p <0.05) and

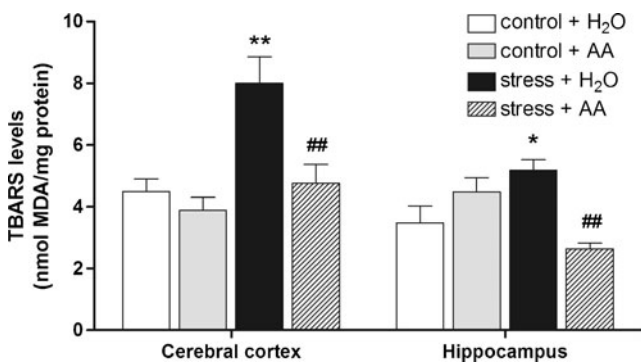


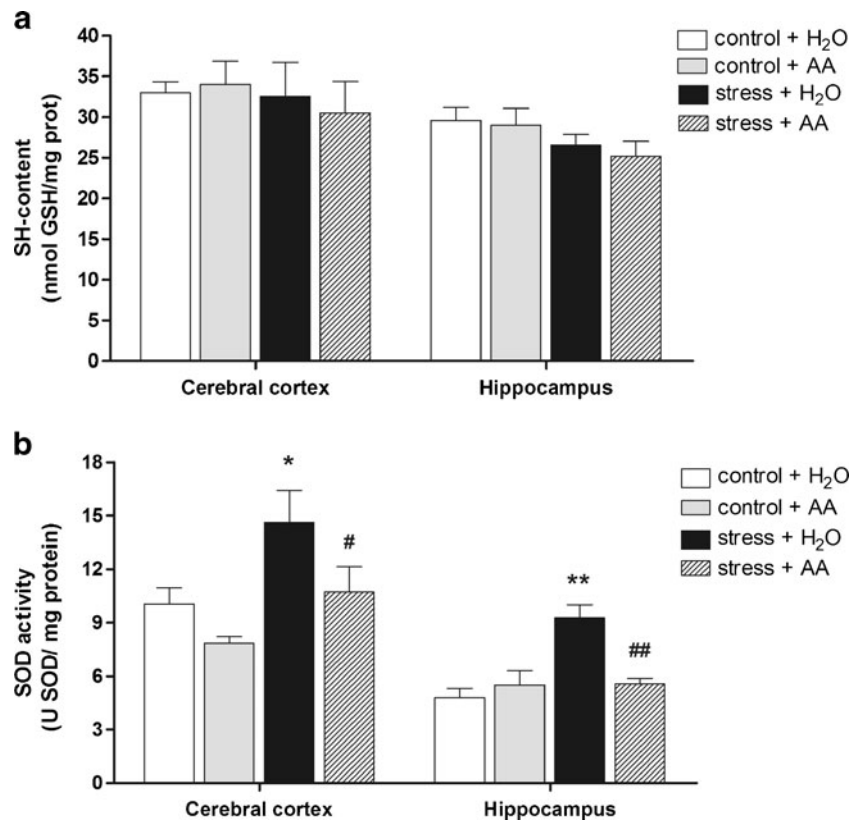
Fig. 2 Effect of treatment with ascorbic acid (1 mg/kg) on thiobarbituric acid reactive substances (TBARS) in the cerebral cortex and hippocampus of mice submitted to acute restraint stress procedure. Bars represent means \pm SEM of six to eight mice. * p <0.05, ** p <0.01 vs. control mice, ## p <0.01 vs. stress + vehicle group

hippocampus (p <0.01) of stressed mice as compared to control mice. These effects induced by restraint stress protocol were significantly blunted by the treatment with ascorbic acid in both encephalic structures: cerebral cortex (p <0.05) and hippocampus (p <0.01). In nonstressed mice, the treatment with ascorbic acid did not cause any change on SOD activity in the evaluated structures, independent on the stress condition.

ROS, such as H₂O₂ (produced from superoxide by SOD), can be detoxified by GSH in a reaction catalyzed by GPx. The GSSG produced in this reaction can be converted back to GSH through a reaction catalyzed by GR, at the expense of the reducing equivalents from NADPH. Both GPx and GR, whose activities are crucial in to the detoxification of peroxides, have been implicated in the etiology of depression. In our experimental protocol, GR activity was increased in the cerebral cortex (p <0.05) of stressed mice, as illustrated in Fig. 4a. This increase was significantly abolished by the treatment with ascorbic acid in stressed mice (p <0.01). In the hippocampus, no significant alterations were observed on GR activity, independent on the stress condition and treatment.

As can be observed in Fig. 4b, stressed mice displayed an increased GPx activity in the cerebral cortex and hippocampus when compared with the control group (p <0.05). This effect was abolished by ascorbic acid treatment in the cerebral cortex (p <0.01). Conversely, ascorbic acid treatment was unable to restore GPx activity in the hippocampus of stressed mice. The administration of ascorbic acid alone did

Fig. 3 Effect of treatment with ascorbic acid (1 mg/kg) on glutathione levels (a) and on SOD activity (b) in the cerebral cortex and hippocampus of mice submitted to acute restraint stress procedure. Bars represent means ± SEM of seven to eight mice for glutathione results and five to six mice for SOD results. ** $p < 0.01$, * $p < 0.05$ vs. control mice, ## $p < 0.01$, # $p < 0.05$ vs. stress + vehicle group



not affect the GPx activity in the nonstressed group in both cerebral structures evaluated.

Western Blot Analyses

Based on reports which indicated a link between cell stressors and abnormal modulation of GSK-3β (He et al. 1998; Wilkinson et al. 2011), we evaluated the phosphorylation of this enzyme in our experimental groups. Figure 5 shows a representative Western blot of the effect of restraint stress and treatment with ascorbic acid on GSK-3β phosphorylation in the cerebral cortex and hippocampus. Densitometric analysis revealed no changes in GSK-3β phosphorylation in the cerebral cortex and hippocampus of mice, independent on stress condition and treatment.

Considering the association between Bcl-2 family proteins, cellular antioxidant defense system, and apoptosis (Kowaltowski and Fiskum 2005), Bax (an apoptosis promoter) and Bcl-2 (an apoptosis inhibitor) levels were measured. No increase in the Bax expression in both cerebral cortex and hippocampus was shown in all the groups, as can be observed in Fig. 6a. As depicted in Fig. 6b, Western blotting analysis of the hippocampus revealed a significant increase in the expression of anti-apoptotic protein Bcl-2 in the stressed group treated with vehicle and ascorbic acid ($p < 0.05$). Likewise, the administration of ascorbic acid to nonstressed mice increased the expression of hippocampal

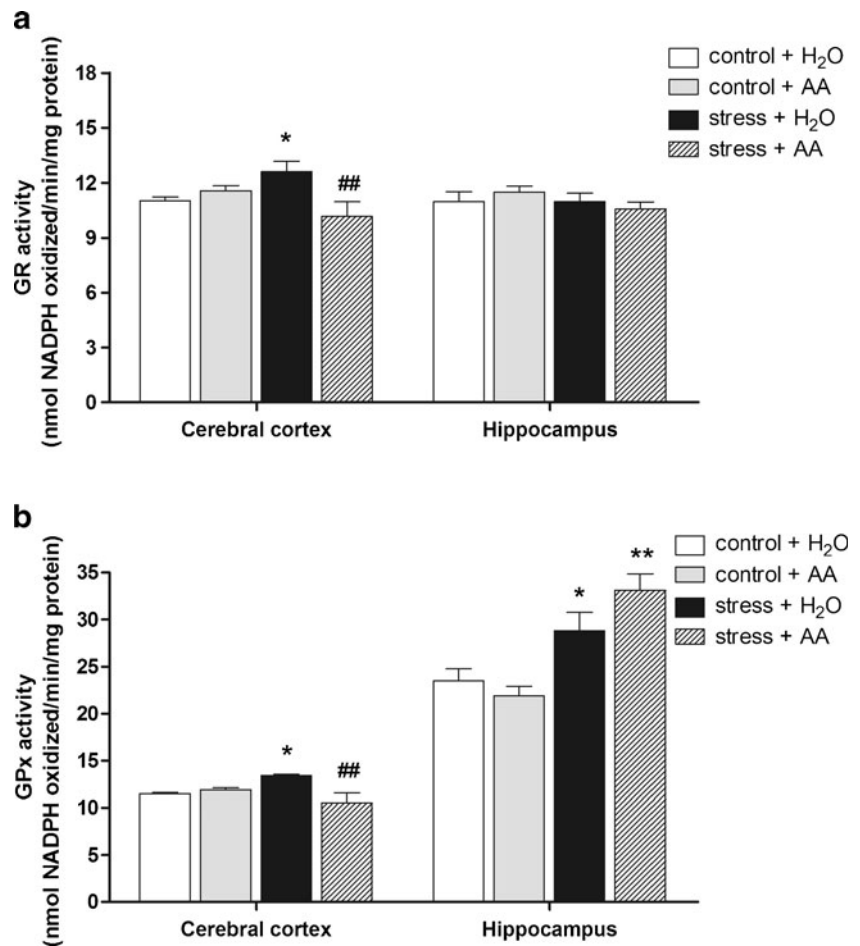
Bcl-2 ($p < 0.01$). In the cerebral cortex, densitometric analysis revealed no changes in Bcl-2 expression, independent on stress condition and treatment.

Discussion

The main findings of the present study can be summarized as follows: (a) Restraint stress procedure in vehicle-treated mice caused a depressive-like behavior, as can be observed in the FST results; (b) a prevention of depressive-like behavior was evident in stressed mice treated with ascorbic acid and fluoxetine; (c) stress procedure induced a prooxidant state which led to an increased lipid peroxidation in the cerebral cortex and hippocampus of vehicle-treated mice; an enhancement in antioxidant defenses was also found in stressed animals, likely as a compensatory response; and (d) ascorbic acid promoted beneficial effects against restraint stress-induced changes in oxidative stress-related parameters in the mouse brain.

There is a complex association among stressful conditions, the mind and body’s response to stress, and the onset of clinical depression (Kioukia-Fougia et al. 2002). Acute immobilization and restraint constitute important models for psychiatric stress since they are inexpensive and involve no bodily harm to the animal subject, once the period of the restraint is terminated. This is a guarantee that any long-term

Fig. 4 Effect of treatment with ascorbic acid (1 mg/kg) on GR activity (a) and on GPx activity (b) in the cerebral cortex and hippocampus of mice submitted to acute restraint stress procedure. Bars represent means \pm SEM of five to six mice. $**p < 0.01$, $*p < 0.05$ vs. control mice, $##p < 0.01$ vs. stress + vehicle group



effects of stress detected are due to the stressor which was applied, rather than to the physical impacts of an irreversible or chronic damage (Buynitsky and Mostofsky 2009).

Mice and rats exposed to emotional stress, including restraint stress at different duration, exhibit depressive-like behavior, as indicated by the increased immobility time,

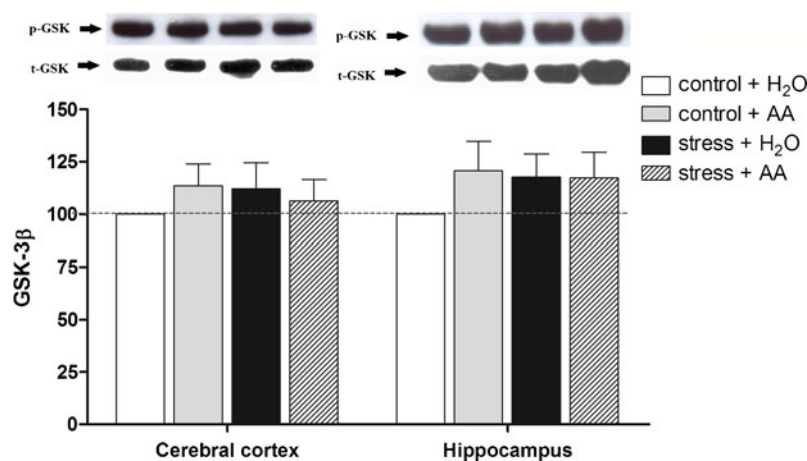
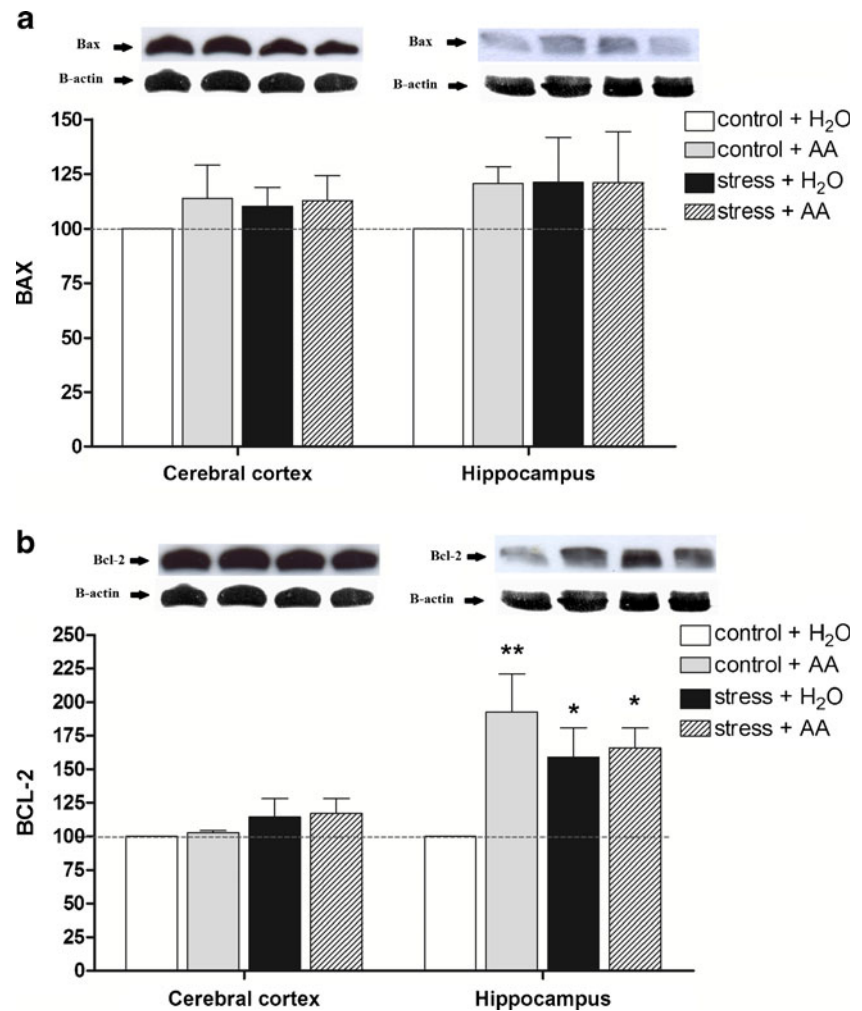


Fig. 5 Effect of treatment with ascorbic acid (1 mg/kg) on the levels of phospho-glycogen synthase kinase-3 β (GSK-3 β) in the cerebral cortex and hippocampus of mice submitted to acute restraint stress procedure. Levels of phospho-GSK-3 β in cerebrocortical and hippocampal homogenates were detected by SDS-PAGE and Western blotting with an anti-phospho-ser9-GSK-3 β antibody. A representative image and

quantitative analysis normalized to the GSK-3 β (upper bands, GSK-3 β ; lower bands, GSK-3 β) bands are shown. The results are expressed as the percentage of vehicle control levels and represent the mean \pm SEM of seven (hippocampus results) or eight (cerebral cortex results) animals per group

Fig. 6 Effect of treatment with ascorbic acid (1 mg/kg) on the expression of Bax (a) and Bcl-2 (b) in the cerebral cortex and hippocampus of mice submitted to acute restraint stress procedure. Expression of Bax and Bcl-2 in the cerebrocortical and hippocampal homogenates was detected by SDS-PAGE and Western blotting with anti-Bax and anti-Bcl-2 antibody. A representative image and quantitative analysis normalized to the β -actin (*upper bands*, Bax or Bcl-2; *lower bands*, β -actin) bands are shown. The results are expressed as the percentage of vehicle control levels and represent the mean \pm SEM of five (Bcl-2 results) or six (Bax results) animals per group. ** $p < 0.01$, * $p < 0.05$ vs. control mice



particularly in the FST, a very specific cluster of stress-induced behaviors (Holmes 2003; Poleszak et al. 2006; Zafir et al. 2009; Capra et al. 2010; Park et al. 2010; Naert et al. 2011). In agreement with these studies, our results showed that acute restraint stress for 7 h increased the immobility time in the FST in mice. Moreover, we demonstrated that restraint stress-induced depressive-like behavior in mice can be prevented by administration of ascorbic acid and fluoxetine. Also, it is important to note that neither stress protocol nor ascorbic acid or fluoxetine treatment affected the spontaneous locomotion, ruling out the hypothesis that the results observed in the FST could be influenced by differences in locomotor behavior.

Considering the clinical studies suggesting that ascorbic acid could be beneficial for treating depression and the involvement of this vitamin in the modulation of mood (Brody 2002; Cocchi et al. 1980), our research group has been interested in studying the antidepressant-like effect of ascorbic in mice. Although the mechanisms responsible for the antidepressant properties of this compound is not fully understood, our published data indicated that it involves an interaction with monoaminergic systems (Binfaré et al. 2009),

an inhibition of either the L-arginine–NO pathway or NMDA receptors (Moretti et al. 2011), and the modulation of neuronal excitability, via inhibition of K⁺ channels (Moretti et al. 2012a), targets that have been suggested to be implicated in the pathophysiology of depression (Harkin et al. 1999; Elhwuegi 2004). Moreover, our group recently demonstrated that repeated ascorbic acid treatment, similar to fluoxetine, reversed depressive-like behavior (assessed in the TST and in the splash test) induced by chronic unpredictable stress in mice (Moretti et al. 2012b), particularly interesting data since this procedure has been suggested to be closely related with depressive symptoms in humans.

It is important to be mentioned that Binfaré et al. (2009) revealed that when administered systemically or centrally in mice, ascorbic acid elicited antidepressant-like effect in the TST, but not in the FST, probably due to potentially different substrates and neurochemical pathways mediating mice performance in the FST as compared to the TST (Bai et al. 2001). Despite the effect of ascorbic acid in the FST was less effective than that obtained after fluoxetine administration, the results observed in the present study suggest that a single restraint stress is able to induce behavioral alteration in mice

that leads to high sensitivity to the antidepressant action of ascorbic acid in the FST. This finding is in line with studies showing that imipramine and magnesium reversed the depressive-like behavior induced by immobility stress, at subeffective doses, that did not produce antidepressant-like effect in the FST in nonstressed mice (Poleszak et al. 2006; Capra et al. 2010). Another point to be considered is that the antidepressant-like effect of ascorbic acid and fluoxetine in the FST was evaluated 8 h and 40 min after its administration, which may account for the lack of effect of these compounds in control nonstressed mice.

It has been shown that emotional stress may induce oxidative damage and considerably changes the balance between prooxidant and antioxidant factors in the brain. In this study, we investigated the influence of 7 h of physical restraint on markers of oxidative damage to lipids and antioxidant capacity. Our results showed that depressive-like behavior induced by restraint is accompanied by a considerable lipid peroxidation, as supported by increasing amount of TBARS in the cerebral cortex and hippocampus of mice. Indeed, previous studies have already shown that immobilization stress targets the brain for lipid peroxidation, as their levels were found to be highest in this tissue (Sahin and Gumuslu 2007). Consistent with our findings, studies have demonstrated that acute and repeated restraint stress induces an increase in TBARS levels in the hippocampus of rats and mice (Fontella et al. 2005; Ahmad et al. 2012; García-Fernández et al. 2012). Accordingly, in humans, serum MDA was found to be increased in the plasma and serum of depressive patients as compared to control subjects (Bilici et al. 2001; Khanzode et al. 2003).

The present study also found an increase in SOD (cerebral cortex and hippocampus), GR (cerebral cortex), and GPx (cerebral cortex and hippocampus) activities in stressed mice, indicating an alteration in antioxidant brain defenses following acute restraint stress. Supporting these findings, preclinical studies showed that acute and chronic protocols of restraint stress induce an increase in SOD, CAT, and GPx activities in the brain of mice/rats (Fontella et al. 2005; Pajović et al. 2006; Balk et al. 2010, García-Fernández et al. 2012). The increased activities of the antioxidant enzymes in response to restraint stress shown in our study are also consistent with clinical findings indicating that depressed patients have significantly higher activities of GR, GPx, SOD, and CAT than those of healthy controls (Bilici et al. 2001; Galecki et al. 2009a).

Evidence indicates that exposure to ROS activates nuclear factor (erythroid-derived 2)-like 2 (Nrf2), a primary transcriptional regulator of a majority of the antioxidants including dismutases (SODs) (McCord and Edeas 2005), catalase (Dringen et al. 2005), GR, and GPx (Mills 1957; Osburn and Kensler 2008). The exact mechanism involved in the stress-induced increase in the activities of antioxidant enzymes was not investigated here; however, it is plausible to suppose that it

may be dependent on Nrf2 activation resulting from an elevated rate of formation of ROS in the hippocampus and cerebral cortex of mice. Although this idea is in agreement with recent lines of evidence that point to Nrf2 activators as promising therapeutic strategies to treat depression (for a review, see Maes et al. 2012), it represents a very new research topic that needs further investigation.

Importantly, the cerebral cortex and hippocampus represent different cell types and have, at least in part, particularities regarding their physiological and metabolic characteristics, including differences in their antioxidant capacity (Prediger et al. 2004), cellular oxidative metabolism, production of compounds which can lead to enhanced formation of free radicals (Gamero et al. 2003; Graumann et al. 2002), or heterogeneity in the distribution of iron in the brain of mice (Benkovic and Connor 1993). This is particularly important to explain the regional differences observed in the vulnerability of GR activity to stress. Distinct responses of different brain structures in the vulnerability to stress effects and treatments were also reported by other authors (de Vasconcellos et al. 2006; Manoli et al. 2000; Prediger et al. 2004), as well as by previous studies from our group (Brocardo et al. 2007; Trevisan et al. 2008; Moretti et al. 2012b).

Ascorbic acid has many nonenzymatic actions and is a powerful water-soluble antioxidant. It protects low density lipoproteins from oxidation and reduces harmful oxidants in the CNS. Previously, we demonstrated that repeated administration of ascorbic acid, which prevented chronic unpredictable stress-induced depressive-like behavior, also restored the stress-induced lipid peroxidation in the cerebral cortex and hippocampus of mice (Moretti et al. 2012a), suggesting a potential relationship between both events. In the present study, we have examined whether the pretreatment with ascorbic acid can protect against lipid peroxidation induced by acute restraint stress. Here, we extend our previous findings by indicating that one administration of ascorbic acid restored the stress-induced lipid peroxidation in both the hippocampus and cerebral cortex, suggesting that ascorbic acid can exert a protective effect in a short period of pretreatment. Consistent with our result, Santos et al. (2009) showed that lipid peroxidation in the hippocampus during experimental seizures significantly ameliorates with the administration of ascorbic acid. Moreover, addition of ascorbate to cultured cells, brain slices, and brain microsomes prevents lipid peroxidation induced by different oxidizing agents (Seregi et al. 1978; Kovachich and Mishra 1983).

Interestingly, ascorbic acid treatment, which prevented restraint stress-induced cerebrocortical and hippocampal lipid peroxidation, also prevented the increase of SOD activity in both encephalic structures. Moreover, ascorbic acid inhibited the increased GR and GPx activities in the cerebral cortex of animals submitted to stress protocol. Based on studies which suggest increased antioxidant enzymes activity as an indicative

oxidative stress in depressed patients (Bilici et al. 2001; Michel et al. 2007; Galecki et al. 2009a, b; Selek et al. 2008), the observed effect of ascorbic acid in stressed mice might indicate that this vitamin blunted primary prooxidative stimulus induced by acute restraint stress.

The association between mood disorders and neuronal stress, as well as oxidative stress, is well-established (Andreazza et al. 2008; Ng et al. 2008; Steckert et al. 2010). Cell stressors can contribute to abnormal modulation of GSK-3 β , an enzyme that may reduce neuronal resilience in stressful environments (He et al. 1998; Wilkinson et al. 2011). Based on these evidences, we took advantage of the restraint protocol, which increases the depressive-like behavior and brain oxidative damage, to evaluate the effects of acute stress on GSK-3 β phosphorylation in the cerebral cortex and hippocampus of mice. GSK-3 β activity is inhibited by phosphorylation at serine⁹, and this inhibition exerts protective effects and increases neuroplasticity (Gould and Manji 2005). In the present study, we found a lack of effect of stress and ascorbic acid treatment on mice cerebrocortical and hippocampal GSK-3 β phosphorylation. Our results are similar to those obtained by Kozlovsky et al. (2002), which reported that rats exposed to acute (1 day), subchronic (6 days), or chronic (14 days) cold restraint stress presented no effect on GSK-3 β protein levels in the frontal cortex. On the other hand, a recent study demonstrated that rats subjected to immobilization stress 6 h/day for 3 weeks had significantly decreased serine⁹ phosphorylated GSK-3 β in the hippocampus (Park et al. 2011). This difference may be attributed principally to the different experimental protocols (acute or chronic) or to the different brain areas examined in these studies.

Here, we also evaluated the effect of acute restraint stress and treatment with ascorbic on expression of pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2. Under normal homeostatic conditions, individual cells make the decision to live or die based upon the balance of pro- versus anti-apoptotic molecules present and functioning within the cell. In our experimental paradigm, we showed no effect on Bax expression (cerebral cortex and hippocampus) and an increased expression of Bcl-2 protein in the hippocampus, independent on stress condition and treatment. The increased Bcl-2 expression is usually associated with enhanced antioxidant defenses and increased mitochondrial redox capacity (Rudin et al. 2003; Kowaltowski and Fiskum 2005). In addition to decreasing the sensitivity of mitochondria to oxidant-induced mitochondrial permeability transition, the increased redox capacity can protect cells against oxidative stress through detoxification of ROS via glutathione reductase/peroxidase and other antioxidant systems (Kowaltowski and Fiskum 2005). Here, the increased Bcl-2 expression may be a compensatory response to the elevated rate of formation of free radicals in stressed animals. This assumption is

corroborated by data which showed that the reinforcement of the antioxidant defenses observed in Bcl-2 overexpressing cells may be a compensatory response to higher mitochondrial ROS release (Kowaltowski et al. 2004). Of note, the increased Bcl-2 expression in animals treated with ascorbic acid provides further insights into an alternative mechanism of action of this vitamin evolving Bcl-2 regulation of the cellular redox status. Despite other mechanisms or pathways may be implicated in the therapeutic action of ascorbic acid, the balance of endogenous antioxidant defenses and an up-regulation of Bcl-2 protein seem to be an important mechanism by which ascorbic acid exerts its protective action against acute restraint stress.

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