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Synaptosomal Response to Oxidative Stress: Effect of Vinpocetine

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It has been suggested that reactive oxygen species (ROS) play a role in the neuronal damage occurring in ischemic injury and neurodegenerative disorders and that their neutralization by antioxidant drugs may delay or minimize neurodegeneration. In the present study we examine whether vinpocetine can act as an antioxidant and prevent the formation of ROS and lipid peroxidation in rat brain synaptosomes. After ascorbate/Fe^{2+} treatment a significant increase in oxygen consumption (about 5-fold) and thiobarbituric acid reactive substances (TBARS) formation (about 7-fold) occurred as compared to control conditions. Vinpocetine inhibited the ascorbate/Fe^{2+} stimulated consumption of oxygen and TBARS accumulation, an indicator of lipid peroxidation, in a concentration-dependent manner. The ROS formation was also prevented by vinpocetine. Oxidative stress increased significantly the fluorescence of the probes 2′,7′-dichlorodihydrofluorescein ($DCFH_2$-DA) (about 6-fold) and dihydrorhodamine (DHR) 123 (about 10-fold), which is indicative of intrasynaptosomal ROS generation. Vinpocetine at 100 μM concentration decreased the fluorescence of $DCFH_2$-DA and DHR 123 by about 50% and 83%, respectively. We conclude that the antioxidant effect of vinpocetine might contribute to the protective role exerted by the drug in reducing neuronal damage in pathological situations.

Keywords: Neuroprotection, oxidative stress, synaptosomes, vinpocetine

Abbreviations: BHT, 2,[6]-Di-*t*-butyl-*p*-cresol; $DCFH_2$, 2′,7′-dichlorodihydrofluorescein; DHR, dihydrorhodamine; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances

INTRODUCTION

Oxidative stress has been implicated in numerous pathological processes in the central nervous system (CNS) mainly in ischemia, hypoxia, trauma, aging and in several neurodegenerative disorders such as, Parkinson's disease and Alzheimer's disease.[1–6] Oxidative stress refers to the cytotoxic effects of reactive oxygen species (ROS), such as hydroxyl radical, superoxide anion, and hydrogen peroxide, which may attack cellular components including nucleic acids, proteins and membrane phospholipids promoting neuronal degeneration.[2,7–9]

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Several mechanisms are known to be operative in CNS tissue injury induced by ischemia and reperfusion/reoxygenation.[10] The excessive stimulation of glutamate receptors, caused by an extracellular accumulation of excitatory amino acids, glutamate playing a predominant role, leads to intracellular accumulation of high levels of Ca^{2+}, mitochondrial dysfunction and the subsequent formation of ROS leading to lipid peroxidation.[11–14] Previous studies have demonstrated that the presence of N-methyl-D-aspartate (NMDA) antagonists, free radical scavengers and antioxidants protect neurons in CNS under ischemic conditions.[15–17]

Vinpocetine (ethyl-apovincamine, 22-oate), a vincamine derivative, is a drug clinically useful in the treatment of various cerebrovascular diseases (Figure 1). It has been documented that the drug reduces neuronal damage induced by hypoxia[18] or ischemia,[19] inhibits the cytotoxic effect of glutamate in cerebrocortical neurons[20] and the voltage-dependent Na^+ and Ca^{2+} channels,[21–23] and has anticonvulsant properties.[24] However, little is known about its antioxidant characteristics in brain preparations. The neuroprotective effect of vinpocetine in ischemic conditions could be explained, at least partially, in terms of its possible antioxidant effect.

In the present study, the possible protective role of vinpocetine against ROS formation and lipid peroxidation, was evaluated in synaptosomes treated with ascorbate/Fe^{2+}. The oxidant pair ascorbate/Fe^{2+} has heen shown to be effective as a membrane peroxidative agent in synaptosomal preparations and is also active in the formation of free radicals.[25,26]

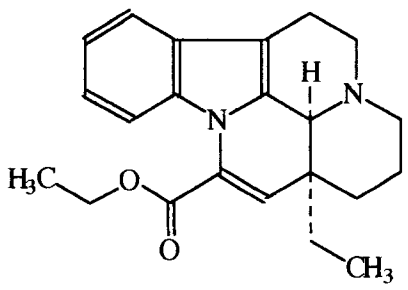

FIGURE 1 Chemical structure of vinpocetine.

MATERIALS AND METHODS

Materials

All chemicals used were of analytical grade and were obtained from Sigma Chemical Co., USA. $DCFH_2$-DA and DHR 123 were obtained from Molecular Probes (USA). Vinpocetine (minimum 98%), a kind gift of Tecnimede (Lisbon, Portugal), was first dissolved in a few drops of 1 M HCl, diluted with standard medium and then the solutions neutralized with 1 M NaOH.

Preparation of Synaptosomes

Crude synaptosomes were prepared from brain of male Wistar rats (180–220 g), according to the method of Hajós,[27] with some modifications. After animal decapitation, the whole cerebral cortices were rapidly removed and homogenized in 10 volumes of 0.32 M sucrose buffered at pH 7.4 with Tris. The homogenate was centrifuged at 1000 g for 10 min and the synaptosomes were isolated from the supernatant by centrifugation at 12,000 g for 20 min. The white and fluffy synaptosome layer was then resuspended, respun and resuspended in the sucrose medium at a protein concentration of 15–20 mg/ml, as determined by the biuret method. Experiments were carried out within 3 h of preparation.

Induction of Oxidative Stress

The oxidizing agents, ascorbic acid and iron (Fe^{2+}, ferrous sulfate) were used to induce oxidative stress. Synaptosomes (1 mg/ml) were peroxidized by incubation at 30°C, in a standard medium containing (in mM): 132 NaCl, 3 KCl, 1 $MgCl_2$, 1 NaH_2PO_4, 1.2 $CaCl_2$, 10 glucose and 20 mM Hepes adjusted to pH 7.4 with Tris,

supplemented with 0.8 mM ascorbic acid and 2.5 μM iron in the presence or in the absence of vinpocetine. Ascorbate and iron solutions were prepared immediately before use and protected from light. Controls were incubated at 30°C during the same period of time, in the absence of ascorbic acid and ferrous sulfate, with or without vinpocetine.

Evaluation of Lipid Peroxidation and Effect of Vinpocetine

The extent of lipid peroxidation was determined by measuring thiobarbituric acid reactive substances (TBARS), expressed as malondialdeyde (MDA), using the thiobarbituric acid assay (TBA), according to a modified procedure described by Ernster and Nordenbrand.[28] The amount of TBARS formed was calculated using a molar coefficient of $1.56 \times 10^5\,mol^{-1}\,cm^{-1}$ and expressed as nmol MDA/mg protein.

The oxygen consumption was measured using a Clark-type electrode (YSI Model 5331, Yellow Springs Inst.) in a closed glass chamber equipped with magnetic stirring, thermostated at 30°C, as described by Dinis *et al.*[29] Reactions were started by the addition of ascorbate–iron. The changes in O_2 tension were recorded in a potentiometric chart recorder and the oxygen consumption calculated assuming an oxygen concentration of 230 nmol O_2/ml. Vinpocetine and Trolox were introduced in the incubation medium before the addition of ascorbate–iron. Blank experiments, in the absence of synaptosomes were performed to evaluate the oxygen consumption rate induced by ascorbate/Fe^{2+} itself.

Monitoring Reactive Oxygen Species Generation and the Effect of Vinpocetine

ROS were measured according to Bass *et al.*[30] by following the oxidation of 2′,7′ -dichlorodihydrofluorescein ($DCFH_2$-DA) to fluorescent DCF, which detects the formation of intracellular peroxides. Synaptosomes (1 mg/ml) were incubated with 5 μM $DCFH_2$-DA, for 15 min, in the standard medium containing (in mM): 132 NaCl, 3 KCl, 1 $MgCl_2$, 1 NaH_2PO_4, 1.2 $CaCl_2$, 10 glucose and 20 mM Hepes adjusted to pH 7.4 with Tris. After washing the DCF fluorescence was measured in the same medium with excitation at 502 nm and emission at 550 nm, using a SPEX fluorog spectrometer equipped with a thermostated water bath. The increments in fluorescence, due to oxidation of $DCFH_2$ to DCF, were recorded for 20 min and expressed as arbitrary units. Basal fluorescence was registered during 2.5 min and then the oxidative stress was induced by the addition of ascorbate 0.8 mM and Fe^{2+} 2.5 μM. Control experiments, in the absence of ascorbate/Fe^{2+}, were performed.

The dye dihydrorhodamine 123 (DHR 123) was also used to quantify the levels of intracellular ROS according to Royall and Ischiropoulos.[31] DHR 123 enters the cell and fluoresces when oxidized by ROS to rhodamine 123 detecting the formation of intrasynaptosomal peroxides. The positive charged rhodamine moves to the inside negative mitochondrial environment, being suggested as a probe to measure mitochondrial ROS production. Synaptosomes (1 mg/ml) were incubated with 5 μM DHR, for 20 min, in the standard medium. After washing, the rhodamine fluorescence was measured in the same medium, with excitation at 500 nm and emission at 536 nm, as described for DCF, and expressed as arbitrary units. Basal fluorescence was registered during 2.5 min and then the oxidative stress was induced by the addition of ascorbate 0.8 mM and Fe^{2+} 2.5 μM. Control experiments, in the absence of ascorbate/Fe^{2+}, were performed.

Metal Binding

The possible interaction of ferrous ions with vinpocetine was estimated by the decrease in the maximal absorbance of iron (II)–ferrozine complex.[32] Briefly, 100 μM of vinpocetine was incubated with 20 μM Fe^{2+} (ammonium ferrous

sulfate) in 5% ammonium acetate, pH 6.9. The reaction was initiated by the addition of 100 μM ferrozine and the absorbance at 562 nm was read at 15 min.

Data Analysis

Data are expressed as mean ± SEM of the indicated number of experiments. The significance of the differences between the means was calculated by the unpaired Student's *t*-test or by the one-way ANOVA plus Student–Newman–Keuls post-test for multiple comparisons. P values of ≤ 0.05 were considered to represent significant differences.

RESULTS

Lipid Peroxidation and the Effect of Vinpocetine

The oxygen consumption and TBARS assay were used to determine the level of lipid peroxidation induced in synaptosomes by ascorbate/Fe^{2+}. Figure 2 shows that the time-course of accumulation of TBA reacting compounds. The maximal values of oxygen consumption and TBARS accumulation, observed at 15 min of peroxidation, were 240.17 ± 26.04 nmol/mg protein and 21.44 ± 1.51 nmol MDA/mg protein, respectively. Inhibition of oxygen consumption and TBARS formation was observed in the presence of butylated hydroxy toluene (BHT), a known anti-lipid peroxidative reagent. The amount of TBARS formed was negligible and a slow oxygen consumption was observed in control experiments in the absence of ascorbate/Fe^{2+}. When oxygen consumption was plotted against the amount of TBARS accumulated (Figure 3), a good correlation was observed (correlation coefficient: $r = 0.98$), suggesting that, in our experimental conditions, the extent of lipid peroxidation can be monitored continuously by changes in the oxygen consumption.

We determined if vinpocetine could act as an antioxidant, through the scavenging of peroxyl

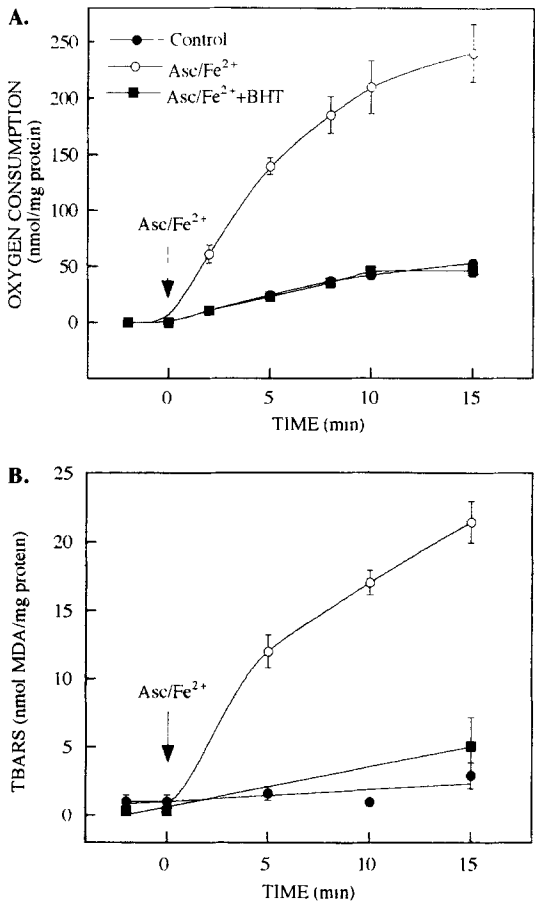

FIGURE 2 Time-course of oxygen consumption (A) and accumulation of TBARS (B) by rat brain synaptosomes submitted to oxidative stress induced by ascorbate 0.8 mM and iron 2.5 μM. Incubation was carried out at 30°C. Control experiment (●) represent the spontaneous O_2 consumption or TBARS formation in the absence of ascorbate–iron. The concentration of BHT was 0.01%. Data are the mean ± SEM of three to four different experiments.

radicals formed after synaptosomal oxidation induced with ascorbate/Fe^{2+}, by measuring its effects on oxygen consumption and TBARS formation. Figure 4A displays the inhibition of lipid peroxidation as a function of drug concentration. Increasing the concentration of vinpocetine up to 100 μM induced a dose dependent decrease in oxygen consumption. In the presence of 100 μM vinpocetine this decrease was 38.74 ± 10%. Vinpocetine also lowered the production of TBARS, although it was not as effective as

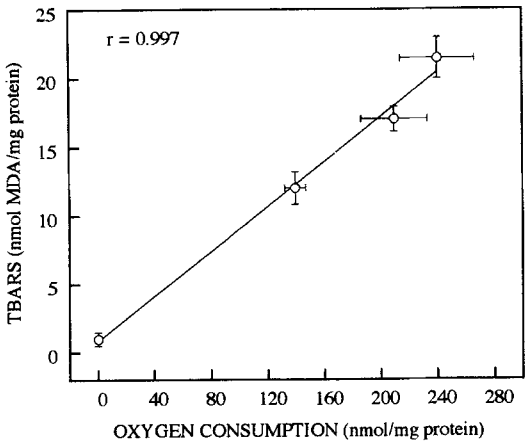

FIGURE 3 Relationship between oxygen consumption and TBARS production in synaptosomes. Data are the mean ± SEM as plotted in Figure 2. Correlation coefficient: $r = 0.997$.

in reducing oxygen consumption (Figure 4B). Whereas 100 μM vinpocetine decreased the oxygen consumption of about 62% in comparison with the control, the same concentration exerted an effect of about 40% in TBARS accumulation. The comparison of the antioxidant capacity of vinpocetine with that of the water-soluble analog of α-tocopherol (Trolox), which is an effective radical scavenger,[33] indicated that both the substances had a similar behavior (Figure 4).

Effect of Vinpocetine in the Formation of Reactive Oxygen Species upon Exposure to Ascorbate/Fe^{2+}

The contribution of oxidative stress to the formation of ROS in our synaptosomal preparation, namely peroxides, is shown in Figure 5. When synaptosomes were submitted to ascorbate/Fe^{2+}, an increased free radical formation occurred as compared to control conditions. At 15 min, the production of peroxides increased by 6-fold (from 4.20 ± 0.24 to 24.04 ± 1.60 arbitrary units of DCF fluorescence). Vinpocetine at 100 μM reduced significantly the formation of peroxides to 12.29 ± 3.43 arbitrary units of fluorescence (Figure 5B).

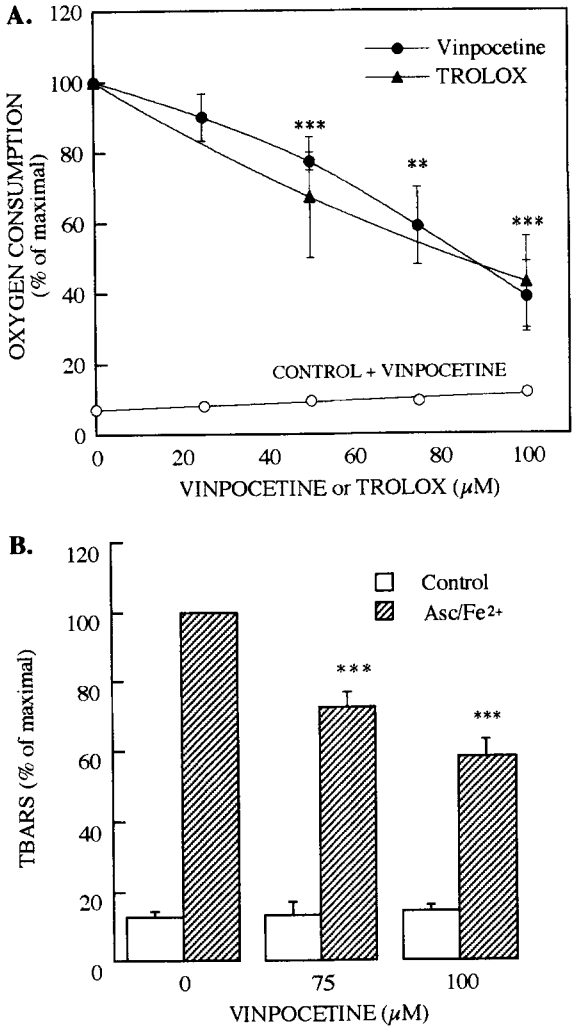

FIGURE 4 Effect of vinpocetine on the oxidative stress induced by ascorbate–iron. Synaptosomes were exposed to 0.8 mM ascorbate and 2.5 μM iron for 15 min at 30°C in the presence or in the absence of vinpocetine. The extent of lipid peroxidation was evaluated by determining the oxygen consumption (A) and the accumulation of TBARS (B). Data expressed as percentage of the maximal are the mean ± SEM of three to seven different experiments. Statistical significance: $^{***}P < 0.001$, $^{**}P < 0.05$ compared with synaptosomes submitted to ascorbate/Fe^{2+} treatment in the absence of vinpocetine.

We also measured the formation of free radicals induced by ascorbate/Fe^{2+} by using the fluorescence probe DHR 123 (Figure 6). Formation of ROS, as measured by following the increments in rhodamine fluorescence, was calculated at 15 min after the addition of ascorbate/Fe^{2+}. ROS levels

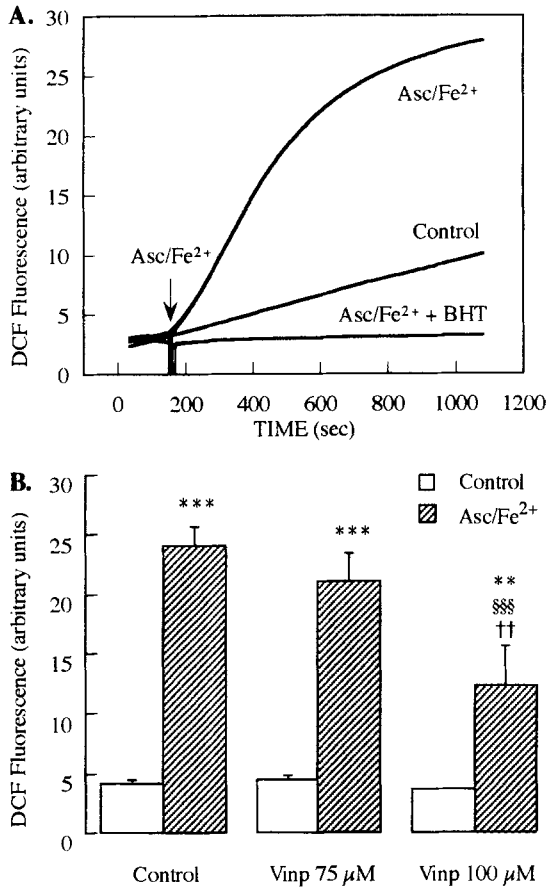

FIGURE 5 Effect of oxidative stress and vinpocetine on the levels of peroxides in synaptosomes. Synaptosomal preparation loaded with 5 μM $DCFH_2$-DA was submitted to oxidative stress induced by 0.8 mM ascorbate and 2.5 μM iron as described in Materials and Methods. (A) Representative experiment of the kinetics of formed ROS. (B) Inhibition of ROS formation as a function of vinpocetine concentration in synaptosomal preparations, measured after 15 min of peroxidation. The concentration of BHT was 0.01%. Data are expressed as the mean ± SEM for five experiments. Statistical significance: $^{***}P < 0.001$, $^{**}P < 0.01$ as compared with the respective control in the presence of vinpocetine; $^{§§§}P < 0.001$ compared with synaptosomes submitted to ascorbate/Fe^{2+}; $^{††}P < 0.01$ as compared with synaptosomes submitted to ascorbate/Fe^{2+} in the presence of 75 μM vinpocetine.

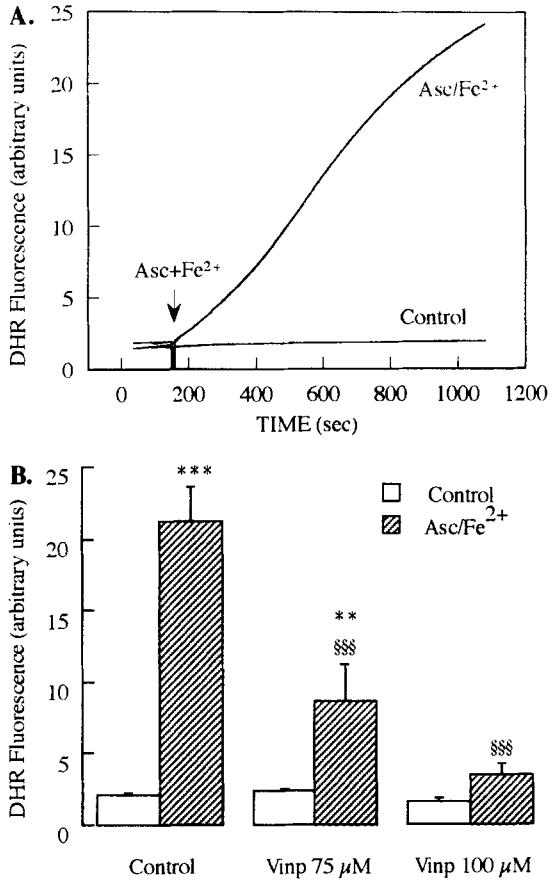

FIGURE 6 Effect of oxidative stress and vinpocetine on the levels of ROS in mitochondria. Synaptosomal preparation loaded with 5 μM DHR was submitted to oxidative stress induced by 0.8 mM ascorbate and 2.5 μM iron as described in Materials and Methods. (A) Representative experiment of the kinetics of formed ROS. (B) Inhibition of ROS formation as a function of vinpocetine concentration in synaptosomal preparations, measured after 15 min of peroxidation. Data are expressed as the mean ± SEM for four to six experiments. Statistical significance: $^{***}P < 0.001$, $^{**}P < 0.05$ as compared with the respective control in the presence of vinpocetine; $^{§§§}P < 0.001$ as compared with synaptosomes submitted to ascorbate/Fe^{2+}.

increased significantly from 2.17 ± 0.14 to 21.31 ± 2.34 arbitrary units of fluorescence, when synaptosomes were submitted to ascorbate/Fe^{2+} (Figure 6B). Vinpocetine at 50 and 100 μM decreased significantly the formation of free radicals in peroxidized synaptosomes to values of 8.63 ± 2.62 and 3.47 ± 0.81 arbitrary units of DHR fluorescence, respectively. The increased formation of ROS in oxidized synaptosomes was prevented by the presence of BHT. In control conditions the levels of free radicals were not affected by the presence of vinpocetine.

Vinpocetine had no apparent Fe^{2+}-binding effect. In the presence of 100 μM vinpocetine the

formation of the complex ferrozine–Fe^{2+} was not affected, suggesting that the ability of vinpocetine to protect synaptosomes from oxidative stress is not the result of the decrease in the concentration of Fe^{2+} necessary to initiate membrane oxidation (data not shown).

DISCUSSION

The present study has provided an insight into the possible protective role of vinpocetine against oxidative stress induced by ascorbate/Fe^{2+}. Evaluation of lipid peroxidation (Figure 4) and ROS formation (Figures 5 and 6) clearly demonstrated that vinpocetine is highly effective against ascorbate/Fe^{2+} induced peroxidation in synaptosomes by inhibiting ROS formation and lipid peroxidation. Vinpocetine effect seems not to involve an iron chelating mechanism induced by the drug.

Ascorbate/Fe^{2+} is thought to induce lipid peroxidation of synaptosomes by inducing the formation of ROS through the iron catalyzed Haber–Weiss reaction.[7,34] These oxygen species are highly reactive and can initiate chain reactions, such as lipid peroxidation than can alter the structure, function and permeability of the membranes, which ultimately result in cellular death. To determine the extent of lipid peroxidation the production of TBARS and oxygen consumption were measured, since the selection of only a single method for monitoring peroxidation could give misleading results.[34] Our results indicate that incubation of synaptosomes with ascorbate/Fe^{2+} increased significantly the consumption of oxygen and the levels of TBARS (Figures 2 and 4) and that there was a good correlation between oxygen consumption and the amount of TBARS formed over a period of 15 min (Figure 3). A similar relationship between both oxygen consumption and TBARS was reported in rat liver mitochondria during ADP/Fe^{2+} promoted lipid peroxidation.[35] Although many criticisms of the TBARS assay have been reported in the literature,[36] it was recently demonstrated that, when correctly used, it may provide a simple, rapid and sensitive method to test the potency of antioxidant compounds in brain preparations.[37]

Intrasynaptosomal ROS production detected with the fluorescent probes $DCFH_2$-DA or DHR 123, indicated the presence of ROS, most probably hydroperoxides, formed in the cytoplasm or derived from the mitochondria. $DCFH_2$-DA is a non-fluorescent molecule that crosses cell membranes and is deacetylated to $DCFH_2$ by intracellular esterases.[31] In the presence of ROS, $DCFH_2$ is oxidized to the high fluorescent compound, DCF.[38] DCF-H was also been proved to be an excellent peroxynitrite marker with the potential to detect peroxynitrite formation within the cells, under conditions of nitric oxide and superoxide simultaneous production.[39,40]

The fluorescent probe DHR 123 is uncharged but is oxidized by ROS to the positively charged rhodamine 123, which preferentially accumulates within mitochondria with little loss to extracellular space.[31] DHR 123 was shown to be oxidized not only by hydrogen peroxide and superoxide,[31,41] but also by other radicals such as peroxynitrite.[42] Our results showed that the formation of free radicals in cytosol and in mitochondria, if we assume that rhodamine detects the formation of peroxides inside the mitochondria, was partially prevented by vinpocetine 100 μM.

In this study we observed that Trolox, the water-soluble analogue of α-tocopherol (a derivative of vitamin E), and vinpocetine were equally effective against oxidative stress induced by ascorbate/Fe^{2+}. Vitamin E, is one of the most important lipid soluble antioxidant systems, acting as a powerful chain-breaking agent through the scavenging of peroxyl and alkoxyl radicals within the membranes.[43] Vitamin E efficiency in protecting against induced injury was documented after ascorbate/Fe^{2+} induced oxidative stress, through a decrease in lipid peroxidation, in retinal cells[44,45] and in synaptosomes.[26,46]

The neuroprotective effect of vinpocetine could also result from preventing the reduction of the Na^+ electrochemical gradient and the release of

excitatory amino acids aspartate and glutamate, that occur under oxidative stress. Previous studies in our laboratory have shown that oxidative stress conditions increased the resting intracellular concentration of Na^+ of retinal cells, as a result of the glutamate receptors activation.[47] Vinpocetine, by blocking the Na^+ channels could ameliorate the excitotoxic neuronal injury due to the excessive Na^+ loading.[48] In fact, experiments in synaptosomes showed that vinpocetine reduced the increase of $[Na]_i$ induced by veratridine, by inhibiting the voltage-dependent Na^+ channels.[23] An inhibition of the voltage-dependent Na^+ currents by vinpocetine was also reported for cultured cerebrocortical neurons.[49]

In vivo the clinical effect of vinpocetine may be in part produced by reducing oxidative stress caused by the high concentration of extracellular glutamate as a consequence of cerebral ischemia, hypoxia and stroke. A number of studies over the last decade have demonstrated that ROS are formed following CNS injury and can be initiated by excitotoxic levels of extracellular glutamate.[12–14] Vinpocetine has been shown to inhibit the release of ACh and dopamine evoked by stimulation of glutamate receptors[50,51] and to protect neuronal cells against glutamate toxicity in a concentration dependent manner.[20] Of particular interest is the observation that the efficacy of various antioxidants, in inhibiting lipid peroxidation, correlates well with their protective actions against glutamate induced cytotoxicity.[20,52] Recently, it has been shown that part of the central action of vinpocetine was related to an increase in the protective effect of adenosine.[53] Adenosine is an endogenous protective agent against excitotoxicity induced by hypoxia/ischemia or hypoglycemia by decreasing the release of glutamate.[54]

In this study the ID_{50} values of vinpocetine in ROS formation induced by ascorbate–iron are only pharmacologically relevant if we consider that 100 μM of vinpocetine could be incorporated into neuronal membranes. In fact, at a physiological pH, the octanol/water partitioning value is about 100,[55] suggesting that the concentration of vinpocetine in biological membranes could be at least 100 times higher than its concentration in an aqueous medium. Since, *in vivo*, the plasma levels of the drug after i.v. administration of 10 mg, extrapolated to zero time, are in the order of 960 ng/ml (2.7 μM),[56] the membrane concentration under these conditions should be 270 μM.

In conclusion, vinpocetine can be considered as an antioxidant able to reduce ROS formation and lipid peroxidation, protecting the cellular membranes from the attack of free radicals. Our data, indicating that vinpocetine may function as an antioxidant in protecting synaptosomes from oxidative stress induced by ascorbate–iron, agree with the clinical data showing that vinpocetine is a drug useful in the treatment of various cerebrovascular diseases, when the production of ROS and lipid peroxidation occurs at a high extent. The antioxidant action afforded by vinpocetine could provide an effective protective role in preventing neuronal cells from oxidative injury.

Acknowledgments

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