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Original Contribution

MITOCHONDRIAL FUNCTION IS DIFFERENTIALLY AFFECTED UPON OXIDATIVE STRESS

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Abstract—The mechanisms that lead to mitochondrial damage under oxidative stress conditions were examined in synaptosomes treated with ascorbate/iron. A loss of membrane integrity, evaluated by electron microscopy and by LDH leakage, was observed in peroxidized synaptosomes and it was prevented by pre-incubation with vitamin E (150 μM) and idebenone (50 μM). ATP levels decreased, in synaptosomes exposed to ascorbate/iron, as compared to controls. NADH-ubiquinone oxidoreductase (Cx I) and cytochrome c oxidase (Cx IV) activities were unchanged after ascorbate/iron treatment, whereas succinate-ubiquinone oxidoreductase (Cx II), ubiquinol cytochrome c reductase (Cx III) and ATP-synthase (Cx V) activities were reduced by 55%, 40%, and 55%, respectively. The decrease of complex II and ATP-synthase activities was prevented by reduced glutathione (GSH), whereas the other antioxidants tested (vitamin E and idebenone) were ineffective. However, vitamin E, idebenone and GSH prevented the reduction of complex III activity observed in synaptosomes treated with ascorbate/iron. GSH protective effect suggests that the oxidation of protein SH-groups is involved in the inhibition of complexes II, III and V activity, whereas vitamin E and idebenone protection suggests that membrane lipid peroxidation is also involved in the reduction of complex III activity. These results may indicate that the inhibition of the mitochondrial respiratory chain enzymatic complexes, that are differentially affected by oxidative stress, can be recovered by specific antioxidants.   © 1998 Elsevier Science Inc.

Keywords—Lipid peroxidation, Synaptosomes, Free radicals, Mitochondria respiratory chain, Antioxidants

INTRODUCTION

Oxidative stress has been implicated in many pathological situations occurring in the Central Nervous System (CNS), namely in neurodegenerative disorders, such as Parkinson's and Alzheimer's diseases, and in the physiological process of aging [1,2]. Nervous tissue is very susceptible to oxidative stress, due to its high rate of oxygen consumption, relatively low levels of antioxidant defenses, and a high content of polyunsaturated fatty acids and transition metal ions [3,4]. The increased formation and release of reactive oxygen species (ROS), induces structural and functional alterations of cellular membranes [5]. These alterations are promoted by the attack of free radicals to membrane polyunsaturated fatty acids [6], proteins and DNA [2].

The cells contain a number of antioxidant defenses

that protect them from oxidative insults. These defenses can be provided by specific enzymes, like superoxide dismutase, glutathione peroxidase and catalase and also by non-enzymatic compounds like vitamin E and glutathione [1,6,7]. Mitochondria is the major source of ROS [8], because ubiquinones and cytochrome b family (in complex III) do not retain the partially reduced oxygen intermediates in their active sites, as occurs with cytochrome aa_3, resulting in the electrons transfer directly to oxygen with the formation of the superoxide radical [9]. During oxidative stress free radical production is increased, leading to an alteration of the chemical and physical state of the mitochondrial inner membrane (MIM), where ubiquinone redox cycling operates [10]. The alteration of the phospholipid structure in the mitochondrial inner membrane compromises the functionality of the electron transport chain (ETC), leading to energy failure and eventually to cell death [11].

In the present study we analyzed the effect of oxidative stress, induced by ascorbate/iron, on the activity of the mitochondrial respiratory chain complexes in rat

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brain cortex synaptosomes. The viability of the synaptosomes was analyzed by electron microscopy and LDH leakage. Adenine nucleotide levels were evaluated in order to establish the functionality of mitochondrial ETC. In addition, the effects of vitamin E, idebenone and reduced glutathione (GSH), in preventing the decrease of the activity of mitochondrial respiratory chain complexes, induced by oxidative stress, were analyzed. The predominant involvement of lipid peroxidation and protein oxidation in the impairment of mitochondrial respiratory chain activity was also evaluated.

MATERIALS AND METHODS

Animals, chemicals and reagents

The animals used in these studies were male Wistar rats, 1.5 months old, supplied with normal laboratory diet. All chemicals used were of analytical grade and were obtained from Sigma, (USA). The following compounds were obtained as generous gifts: CoQ_1 and CoQ_2 from Eisai Chemical Company, Tokyo, Japan, and Idebenone from Seber, Portugal.

Isolation of synaptosomes

Synaptosomes were prepared from rat brain cortex homogenates, according to the method of Hajós [12], slightly modified [13]. The synaptosomal pellets were washed and resuspended in buffered sucrose medium at 4°C. Synaptosomal protein concentrations were measured by the Biuret method [14], using bovine serum albumin as standard.

Induction and quantification of lipid peroxidation

Ascorbic acid and Fe^{2+} were used to induce lipid peroxidation [15] and the extent of the peroxidative process was determined by the thiobarbituric acid test (TBA) [16]. Synaptosomes (1 mg/ml of protein, final concentration) were incubated at 30°C for 15 min, in Na^+-medium containing: 132 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 10 mM Hepes-Tris, pH 7.4, with 0.8 mM ascorbic acid and 2.5 μM Fe^{2+}, in the presence or in the absence of antioxidants. Controls were incubated at 30°C during the same period of time, in the absence of ascorbic acid and ferrous sulfate, with or without antioxidants.

The oxidative protectors, vitamin E (150 μM) and idebenone (50 μM), were preincubated for 20 or 15 min, respectively, whereas glutathione (250 μM) was added simultaneously with the oxidant agents, ascorbate and iron. Stock solutions of vitamin E and idebenone were prepared in 100% ethanol. The final concentration of

ethanol (0.8% v/v) present in the experimental assay was shown to have no effect by itself. After induction of lipid peroxidation, synaptosomes were centrifuged and the pellets were washed and resuspended in Na^+ medium. Control and peroxidized synaptosomes were frozen in liquid nitrogen and stored at -80°C for further enzymatic analyzes.

LDH measurements

Synaptosomes plasma membrane integrity was analysed by monitoring the activity of the cytoplasmic enzyme LDH in the extracellular incubation medium. LDH activity was measured spectrophotometrically, according to the method of Bergmeyer and Brent [17], by following the rate of conversion of NADH to NAD^+, at 340 nm. LDH leakage was expressed as a percentage of total LDH activity, which corresponds to the activity determined in the supernatant plus that determined after resuspension of synaptosomal pellets in a hypotonic solution containing 15 mM Tris, pH 7.4.

Electron microscopy

Synaptosomal crude fraction was fixed by the addition of Karnousky reagent (phosphate-buffered 0.1 mol/L, pH 7.2 and 2.5% glutaraldehyde solution), during 1 hour at 4°C. The fixed pellets were then washed with 100 mM sodium cacodylate buffer (pH 7.2). Pellets were included in 1% Agar and then dehydrated in grade ethanol and embedded in Spur. The ultrathin sections were cut with an LKB ultra-microtome ULTROTOME III, then contrasted with uranyl acetate and with lead citrate for transmission electron microscopy. Electron microphotographs were taken with a JEOL JEM-100 SX electron microscope operated at 80 kV.

Analysis of adenine nucleotides

After the incubation period, 1 mg protein was extracted, in ice, with 1.2 M perchloric acid. The acid extracts were centrifuged at $14,000 \times g$ for 1 min. The resulting supernatants were then neutralized with KOH/Tris and stored at -80°C for further analysis. Adenine nucleotides (ATP, ADP and AMP) were assayed by separation in a reverse-phase HPLC, as described by Stocchi et al. [18]. The chromatographic apparatus used was a Beckman System Gold, consisting of a 126 Binary Pump Model and a 166 Variable UV detector, controlled by computer. The column used was a Lichrospher 100 RP-18 (5 μm) from Merck (Germany). An isocratic elution with 100 mM KH_2PO_4 buffer at pH 7.4 and 1% methanol, was performed at a flow rat of 1.2 ml/min. The

adenine nucleotides (ATP, ADP and AMP) were detected at 254 nm, for 6 min.

Citrate Synthase assay

Citrate Synthase activity was measured according to the method of Coore et al. [19], which couples CoA to Ellman's reagent [5,5′-dithiobis (2-nitrobenzoic acid), DTNB]. The formation of 5-thio-2-nitrobenzoate was monitored at 412 nm. The reaction mixture contained 100 mM Tris, pH 8.0, 200 μM acetyl-CoA, 200 μM DTNB, 0.1% (v/v) Triton X-100, and synaptosomal sample. The assay was initiated by the addition of 100 μM oxaloacetate at 30°C. Results were expressed as nmol/min/mg protein.

NADH-Ubiquinone Oxidoreductase (Cx I) assay

Mitochondria complex I activity was measured using a modification of the method of Ragan et al. [20], determining the decrease in NADH absorbance at 340 nm, that leads to the reduction of ubiquinone (CoQ_1) to ubiquinol. The reaction was initiated by the addition of CoQ_1 (50 μM) to the reaction mixture containing 20 mM potassium phosphate, pH 7.2, 10 mM $MgCl_2$, 0.15 mM NADH, 2.5 mg BSA-FFA, 1 mM KCN, and the synaptosomal sample, at 30°C. After 5 min, rotenone (10 μM) was added and the reaction was registered for a further 5 min. The activity of Cx I, expressed as nmol/min/mg protein, was determined using the rotenone sensitive rate.

Succinate-Ubiquinone Oxidoreductase (Cx II) assay

The activity of complex II was measured using a modified method from Hatefi and Stiggal [21]. The activity of Cx II was measured by following the secondary reduction of a dye (6,6-dichlorophenolindophenol, DCPIP) by the ubiquinol formed; after the reduction of ubiquinone (CoQ_2) the disappearance of DCPIP was monitored at 600 nm. The reaction mixture contained 50 mM potassium phosphate buffer, pH 7.4, 20 mM sodium succinate, 0.1 mM di-K EDTA, 74 μM DCPIP, 1 mM KCN, 10 μM rotenone and the synaptosomal sample. The reaction, performed at 30°C, was initiated by the addition of CoQ_2 (50 μM), and TTFA (2-thenoytrifluoroacetone) was added 5 min later, in order to inhibit Cx II activity. Results were expressed as nmol/min/mg protein, the activity of Cx II being determined by using the TTFA sensitive rate.

Succinate Cytochrome C Oxidoreductase (Cx II/III) assay

Cx II/III activity was measured according to the method of King [22]. This assay measures the appearance of reduced cytochrome c at 550 nm. The reaction mixture contained 0.1 M potassium phosphate, pH 7.4, 0.3 mM di-K EDTA, and 0.1 mM cytochrome c. The synaptosomal sample was pre-incubated at 30°C for 5 min with 20 mM succinate, 1 mM KCN to activate the enzyme, and then added to the reaction mixture. The reaction was performed at 30°C. Antimycin A (0.02 mM) was added to inhibit Cx II/III. Activity of Cx II/III, expressed as nmol/min/mg protein, was that measured by using the antimycin A sensitive rate.

Ubiquinol Cytochrome C Reductase (Cx III) assay

Mitochondria Cx III activity was measured according to the method of Ragan et al. [20]. This enzyme donates electrons from ubiquinol (UQ_2H_2) to cytochrome c, leading to the reduction of cytochrome c that was monitored at 550 nm. The enzymatic reaction is of first order and dependent on the concentration of both UQ_2H_2 and cytochrome c. The concentrations of these compounds were determined prior to the assay. The reaction mixture contained 35 mM potassium phosphate, pH 7.2, 1 mM di-K EDTA, 5 mM $MgCl_2$, 1 mM KCN, 5 μM rotenone, 15 μM cytochrome c and the synaptosomal sample. The reaction was initiated by addition of substrate, ubiquinol (15 μM). The activity of Cx III was calculated by the pseudo-first order constant K and the results were expressed as K/min/mg protein.

Cytochrome C Oxidase (Cx IV) assay

Cx IV activity was measured according to the method of Wharton and Tzagoloff [23] by evaluating the oxidation of cytochrome c as a decrease in absorbance at 550 nm. Cytochrome c was reduced with a few crystals of ascorbate, mixed and placed into a dialysis membrane for 18–24 h against 0.01 M phosphate buffer, pH 7.0, at 4°C. Reduced cytochrome c concentration was then determined with 0.1 M ferricyanide. The reaction mixture contained 0.01 M potassium phosphate, pH 7.0 and 50 μM reduced cytochrome c. The reaction was initiated by the addition of the synaptosomal sample, at 30°C. The pseudo first order rate constant K was calculated, because the reaction is of first order with respect to cytochrome c and the results were expressed as K/min/mg protein.

Mitochondria ATP-Synthase (Cx V) assay

The enzymatic activity of ATPase in the mitochondrial inner membrane in synaptosomes was monitored at

660 nm, according to the method of Taussky and Shorr
[24]. Synaptosomes (0.1 mg protein) were incubated in 1
ml of Na^+ medium at 30°C, during 10 min in the
presence or absence of oligomycin, 1 μg/mg protein. The
reaction was initiated by the addition of 1 mM ATP-
Mg^{2+} at pH 7.4, and the synaptosomes were incubated
for further 15 min at 30°C. The reaction was stopped by
the addition of ice cold 40% TCA. Protein was pelleted
by centrifugation at 3,000 $\times$ g, during 5 min. The
absorbance in the supernatant was measured at 660 nm,
5 min after the addition of molibdate reagent. The
amount of P_i produced was determined using a phos-
phate standard curve. Results were expressed as nmol
P_i/mg protein. The difference between the activity de-
termined in the presence or in the absence of oligomycin,
corresponds to the ATP-synthase activity.

Protein oxidation determination

Protein carbonyl content was determined as described
by Levine et al. [25], with slight modifications (Cardoso
et al., personal communication). Synaptosomal pellets (1
mg) were incubated with 0.5 ml of 10 mM DNPH in 2 N
HCl (or 2 N HCl alone for the blanks), for 1 h at room
temperature. The protein hydrazone derivatives were
precipitated with 0.5 ml of 20% trichloroacetic acid, and
the precipitates were washed three times with 1 ml eth-
anol:ethylacetate (1:1). During each washing, the ho-
mogenized pellet was vortexed and left in the washing
solution for 10 min before centrifugation. The final pellet
was resuspended and incubated in 6 M guanidine HCl,
for 15 min at 37°C. The carbonyl content was determined
spectrophotometrically at 360 nm on the basis of molar
absorbance coefficient of 22,000 M^{-1} cm^{-1}.

Data analysis

Data were expressed as means $\pm$SE of the indicated
number of determinations, from at least three indepen-
dent experiments. Statistical significance analysis was
determined using the two-tailed Student's *t*-test (a *p*
value of <.05 was considered significant).

RESULTS

Influence of ascorbate/iron on lipid peroxidation

Synaptosomes exposed to 0.8 mM ascorbate/2.5 μM
iron, showed an extent of lipid peroxidation of 18.03 $\pm$
0.88 nmol TBARS/mg protein, significantly different
from that determined in control synaptosomes, incubated
in the absence of the oxidant pair (2.62 $\pm$ 0.29 nmol
TBARS/mg protein) (Table 1). When synaptosomes
were preincubated with 150 μM vitamin E, for 20 min,

Table 1. Effect of Ascorbate/Iron on the Production of TBARS

	Levels of TBARS (nmol/mg of protein)
Control	2.62 ± 0.29
Ascorbate/iron	18.03 ± 0.88[c]
Control + vit E	2.84 ± 0.16
Ascorbate/iron + vit E	10.57 ± 1.55[b,e]
Control + IDB	2.76 ± 0.26
Ascorbate/iron + IDB	2.74 ± 0.317[f]
Control + GSH	4.64 ± 0.1[h]
Ascorbate/iron + GSH	12.51 ± 1.18[b,e]

Synaptosomes were submitted for 15 min to ascorbate (0.8 mM)/iron
(2.5 μM), and pre-incubated with or without antioxidants, like vitamin
E (vit E, 150 μM), idebenone (IDB, 50 μM), and GSH (250 μM). The
extent of lipid peroxidation, measured by TBARS production, was
determined in synaptosomal pellets as described in Materials and
Methods. Data are expressed as mean $\pm$ SEM for 10–20 independent
preparations. [a]Significantly different when compared to the appropriate
control ([b]$p < .01$, [c]$p < .001$). [d]Significantly different when compared
to peroxidized synaptosomes ([e]$p < .01$, [f]$p < .001$). [g]Significantly
different when compared to control synaptosomes ([h]$p < .01$).

TBARS production determined after incubation with
ascorbate/iron was 10.57 $\pm$ 1.55 nmol TBARS/mg pro-
tein, this value being statistically higher than that of
controls pre-incubated with vitamin E (2.84 $\pm$ 0.16 nmol
TARS/mg protein), but lower than that of peroxidized
synaptosomes, incubated only in the presence of ascor-
bate/iron (18.03 $\pm$ 0.88 nmol TBARS/mg of protein).

When synaptosomes were pre-incubated with 50 μM
idebenone, in the absence of ascorbate/iron, the extent of
lipid peroxidation was 2.76 $\pm$ 0.26 nmol TBARS/mg
protein. In the presence of ascorbate/iron, after pre-incu-
bation with idebenone, the level of TBARS was similar
to that observed in the absence of the oxidant pair,
2.74 $\pm$ 0.32 nmol mg protein, a value that is statistically
different from that determined in peroxidized synapto-
somes (18.03 $\pm$ 0.88 nmol TBARS/mg of protein).

Glutathione did not completely protect synaptosomes
from lipid peroxidation. The extent of lipid peroxidation
increased by 1.5-fold in synaptosomes incubated only
with 250 μM GSH, (4.64 $\pm$ 0.1 nmol TBARS/mg pro-
tein). However, when synaptosomes were peroxidized in
the presence of GSH, a significant decrease of the extent
of lipid peroxidation was observed as compared with
synaptosomes oxidized in the absence of GSH (12.51 $\pm$
1.18 and 18.03 $\pm$ 0.88 nmol TBARS/mg protein, respec-
tively).

Synaptosomes viability upon lipid peroxidation

Synaptosomes viability was measured by the LDH
leakage to the extracellular medium. A significant in-
crease in LDH leakage, of about 3-fold, was observed in
peroxidized synaptosomes (38.88 $\pm$ 1.91%) as compared
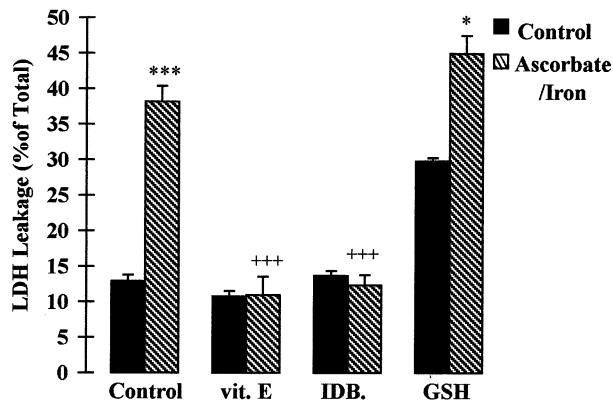to control synaptosomes (12.92 $\pm$ 0.90%) (Fig. 1). The

Fig. 1. Effect of oxidative stress on the LDH-leakage in synaptosomes exposed to 0.8 mM ascorbate and 2.5 μM iron, for 15 min, with or without 150 μM vitamin E (vit E), 50 μM idebenone (IDB) or 250 μM GSH. LDH activity was determined in synaptosomal preparations as described in Materials and Methods. Data are expressed as the mean $\pm$ SEM for 4 independent preparations. *Significantly different when compared to the appropriate control (*$p < .05$, ***$p < .001$). [+]Significantly different when compared to synaptosomes treated with ascorbate/iron ([+++]$p < .001$).

LDH leakage in controls pre-incubated with vitamin E and idebenone was similar to that measured in controls in the absence of antioxidants (10.74 $\pm$ 0.80% and 13.67 $\pm$ 0.71%, respectively). Vitamin E and idebenone protected the LDH release from oxidized synaptosomes (10.96 $\pm$ 2.60% and 12.33 $\pm$ 1.45%, respectively). When synaptosomes were incubated with GSH and ascorbate/iron, no significant decrease in LDH leakage was observed (44.96 $\pm$ 2.50%), as compared with that determined in synaptosomes treated with ascorbate/iron in the absence of GSH. However, the increase in the LDH leakage observed after an ascorbate/iron treatment is reduced in the presence of GSH because when synaptosomes were incubated in the presence of GSH alone, an increase in LDH leakage occurred, as compared to controls (31.50 $\pm$ 5.20% and 13.67 $\pm$ 0.71%, respectively). The morphology of the washed crude synaptosomal fraction was also analyzed by electron microscopy. In synaptosomes incubated in the absence of ascorbate/iron at 30°C, plasma membrane integrity was maintained (Fig. 2A). When synaptosomes were treated with ascorbate/iron, plasma membrane discontinuities were observed as compared to control synaptosomes (Fig. 2B).

Respiratory chain enzyme activities upon oxidative stress

Table 2 shows the activities of mitochondria respiratory chain complexes, expressed relatively to citrate synthase activity to compensate any variation in mitochondria enrichment, and also the actual values of the enzymatic activities. The citrate synthase activity can be
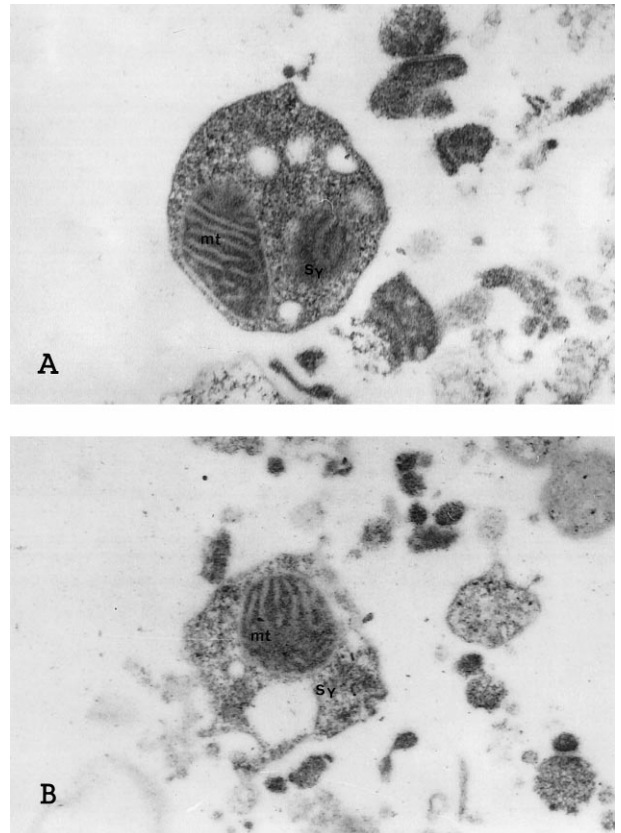

Fig. 2. Electron micrograph of subcellular particles constituting the washed crude synaptosomal pellet. (A) Control synaptosomes. (B) Synaptosomes treated with 0.8 mM ascorbate and 2.5 μM iron, for 15 min, at 30°C. sy (synaptosomes); mt (mitochondria in nerve endings); $\times$63,000.

used for correcting mitochondrial enzyme activities, because it did not change with ascorbate/iron treatment (data not shown). As can be observed in Table 2 there was no significant difference in NADH-Ubiquinone Oxidoreductase (Cx I) and in Cytochrome C Oxidase (Cx IV) activities, when synaptosomes were incubated with ascorbate/iron, but Succinate-Ubiquinone Oxidoreductase (Cx II) activity decreased significantly in peroxidized synaptosomes (0.036 $\pm$ 0.005), as compared with control synaptosomes (0.071 $\pm$ 0.004). The incubation with ascorbate/iron for 15 min inhibited Succinate Cytochrome C Oxidoreductase (Cx II/III) activity for about 2.5-fold (from 0.156 $\pm$ 0.007 in controls to 0.063 $\pm$ 0.004 in peroxidized synaptosomes). Ubiquinol Cytochrome C Reductase (Cx III) activity decreased for about 1.07-fold in oxidized synaptosomes (0.064 $\pm$ 0.006) as compared with controls (0.106 $\pm$ 0.009). ATP-Synthase (Cx V) activity was also inhibited in synaptosomes treated with ascorbate/iron by about 1.87-fold (from 5.62 $\pm$ 0.46 in controls to 3.01 $\pm$ 0.27 in peroxidized synaptosomes). This reduction of mitochondrial respira-

Table 2. Mitochondrial Respiratory Chain Complexes Activities in Synaptosomal Mitochondria

	MRC/Citrate Synthase activity		MRC activity	
	Control	Peroxidized	Control	Peroxidized
Complex I	0.065 ± 0.0046	0.059 ± 0.0041	31.61 ± 2.23	28.69 ± 1.99
Complex II	0.071 ± 0.0044	0.036 ± 0.0055[b]	34.53 ± 2.14	17.51 ± 2.67[b]
Complex II/III	0.156 ± 0.0071	0.063 ± 0.0048[c]	75.86 ± 3.45	30.64 ± 2.33[c]
Complex III	0.106 ± 0.0097	0.064 ± 0.0065[b]	51.55 ± 4.72	31.12 ± 3.16[b]
Complex IV	0.899 ± 0.06	0.868 ± 0.077	437.17 ± 29.18	422.09 ± 37.44
Complex V	5.62 ± 0.468	3.01 ± 0.278[c]	2732.89 ± 227.58	1463.70 ± 135.19[c]

Synaptosomes were exposed to ascorbate (0.8 mM)/iron (2.5 μM) for 15 min, at 30°C. Enzymatic activities were determined as described in Materials and Methods and expressed in terms of nmol/min/mg protein for complexes I, II, V; and in terms of K/min/mg protein for complexes III and IV. Results are expressed as the mean ± SEM for 4–6 independent synaptosomal pellets. [a]Significantly different when compared to control synaptosomes ([b]$p < .01$, [c]$p < .001$).

tory chain complexes activities was statistically significant for the enzymatic complexes analysed.

Effect of oxidative stress on adenine nucleotides levels

The ATP levels in synaptosomes submitted to oxidative stress, decreased for about 1.8-fold (2.63 ± 0.07 nmol/mg of protein in controls; 1.44 ± 0.06 nmol/mg of protein in peroxidized synaptosomes) (Fig. 3), while the levels of ADP and AMP were not significantly affected (data not shown).

The ATP levels in controls pre-incubated with vitamin E, idebenone, GSH, and with all the antioxidants put together, were similar to those measured in controls (1.97 ± 0.0.38, 2.05 ± 0.47, 2.37 ± 0.32 and 3.19 ±
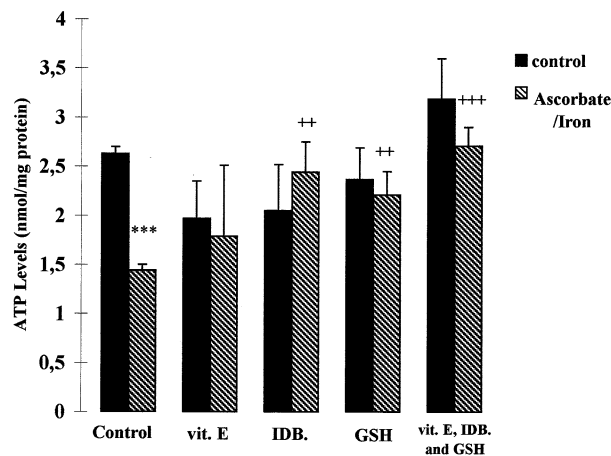

Fig. 3. Effect of oxidative stress and antioxidants on adenine nucleotides levels in synaptosomes exposed to 0.8 mM ascorbate and 2.5 μM iron, for 15 min, with or without 150 μM vitamin E (vit E), 50 μM idebenone (IDB) or 250 μM GSH. ATP levels were determined in synaptosomal preparations as described in Materials and Methods. Data are expressed as the mean ± SEM for 3–5 independent preparations. *Significantly different when compared to the appropriate control (***$p < .001$). +Significantly different when compared to synaptosomes treated with ascorbate/iron (++$p < .01$, +++$p < .001$).

0.41 nmol/mg protein, respectively) (Fig. 3). In the presence of idebenone, GSH and a mixture of all the antioxidants, the ATP levels in oxidized synaptosomes were maintained (2.44 ± 0.31, 2.21 ± 0.24 and 2.71 ± 0.19 nmol/mg protein, respectively). Vitamin E was not efficient on the maintenance of ATP levels in peroxidized synaptosomes (1.79 ± 0.72 nmol/mg protein) (Fig. 3).

Effects of free radical scavengers on Complex II, III, and V activities

When synaptosomes were pre-incubated with 150 μM vitamin E and 50 μM idebenone, the inhibition of complex II activity, measured after ascorbate/iron treatment, remained similar to that observed in the absence of the antioxidants (40% and 50%, respectively), the difference in the value being not statistically significant. In the presence of reduced glutathione (GSH), Complex II activity was almost completely preserved (91.92%) from ascorbate/iron inhibitory effect (Fig. 4A).

When the free radical scavengers vitamin E and idebenone were pre-incubated with synaptosomes, or GSH was added simultaneously with ascorbate/iron, the inhibition of complex III activity was prevented (Fig. 4B). The presence of the free radical scavengers, vitamin E and idebenone, had no protective effect on ATP-synthase activity, an inhibition of 33.68% and 38.13% being observed, respectively. GSH, when incubated simultaneously with ascorbate/iron, almost completely protected ATP-synthase activity (99.82%) (Fig. 4C).

The effect of oxidative stress and free radical scavengers on the formation of Carbonyl Groups

Synaptosomes submitted to 0.8 mM ascorbate/2.5 μM iron shown an increased formation of carbonyl groups, for about 3-fold, as compared to controls (1.56 ± 0.07 and 0.51 ± 0.06 nmol/mg protein, respectively) (Fig. 5).
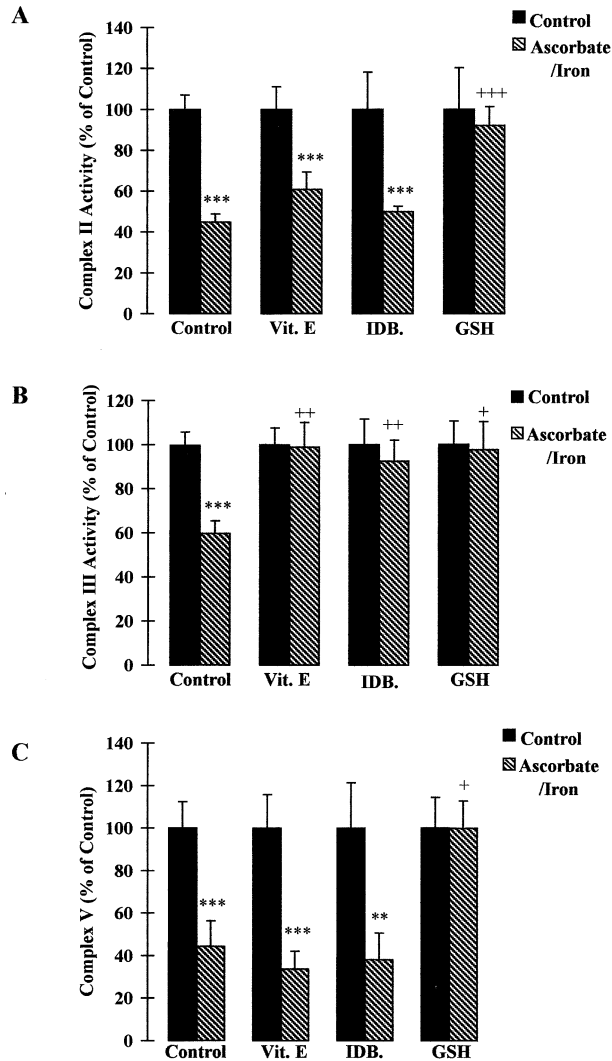
A

B

C

Fig. 4. Effect of oxidative stress and antioxidants on the activity of mitochondrial respiratory chain complexes in synaptosomes exposed to 0.8 mM ascorbate and 2.5 μM iron, for 15 min, with or without 150 μM vitamin E (vit E), 50 μM idebenone (IDB) or 250 μM GSH. (A) The activity of Succinate-Ubiquinone Oxidoreductase (Cx II); (B) The activity of Ubiquinol Cytochrome c Reductase (Cx III); (C) The activity of ATP-synthase(Cx V). Mitochondrial respiratory chain complexes activity was determined in synaptosomal preparations as described in Materials and Methods. Data are expressed as the mean $\pm$ SEM for 4–7 independent preparations. *Significantly different when compared to the appropriate control (**$p < .01$, ***$p < .001$). $^+$Significantly different when compared to synaptosomes treated with ascorbate/iron ($^+p < .05$, $^{++}p < .01$, $^{+++}p < .001$).

The carbonyl content, in oxidized synaptosomes pre-incubated with vitamin E, decreased in comparison to synaptosomes treated with ascorbate/iron (1.19 $\pm$ 0.13, 1.56 $\pm$ 0.07 nmol/mg protein, respectively), but vitamin E did not rescue the carbonyl content to control values (0.56 $\pm$ 0.04 nmol/mg protein). Idebenone prevented the formation of carbonyl groups in peroxidized synaptosomes (0.82 $\pm$ 0.12 nmol/mg protein). When synaptosomes were incubated in the presence of GSH alone, a
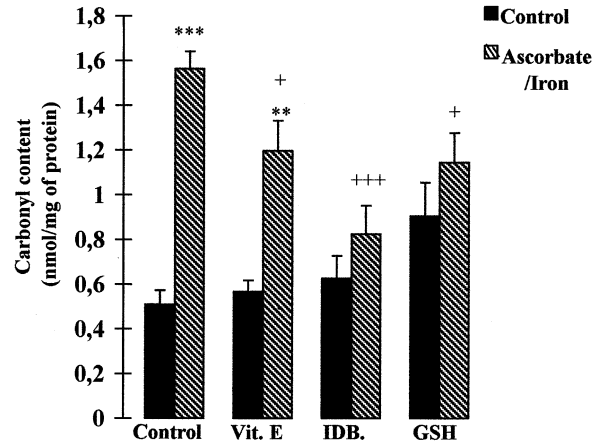

Fig. 5. Effect of oxidative stress and antioxidants on carbonyl content in synaptosomes exposed to 0.8 mM ascorbate and 2.5 μM iron, for 15 min, with or without 150 μM vitamin E (vit E), 50 μM idebenone (IDB) or 250 μM GSH. Carbonyl groups were determined in synaptosomal preparations as described in Materials and Methods. Data are expressed as the mean $\pm$ SEM for 5 independent preparations. *Significantly different when compared to the appropriate control (**$p < .01$, ***$p < .001$). $^+$Significantly different when compared to per-oxidized synaptosomes ($^+p < .05$, $^{++}p < .01$, $^{+++}p < .001$).

small increase in carbonyl content was observed as compared to controls, this increase being statistically not significant (0.90 $\pm$ 0.15 and 0.51 $\pm$ 0.06 nmol/mg protein, respectively). However, a decrease in carbonyl groups occurred in oxidized synaptosomes incubated with glutathione, in comparison to synaptosomes treated only with the oxidant agents (1.14 $\pm$ 0.13 and 1.56 $\pm$ 0.07 nmol/mg protein, respectively).

DISCUSSION

The objective of this work was to define the mechanisms whereby oxidative stress leads to mitochondrial respiratory chain injury. For this purpose, we investigated the effect of ascorbate/iron mediated oxidative stress on the activity of mitochondrial respiratory chain enzymatic complexes in brain cortex synaptosomes. Our results show that ascorbate/iron gives rise to a marked inhibition of complex II, complex III, and complex V activities that can be prevented by the antioxidants vitamin E, idebenone, and GSH.

Ascorbate/iron has been shown to give rise to the highly reactive hydroxyl radical by the iron-catalyzed Haber–Weiss reaction [26,27]. The superoxide anion radical appears to be the first oxygen reduction product generated under both physiological and pathological conditions [8]. Subsequent dismutation of superoxide radical generates hydrogen peroxide and the reaction of H_2O_2 with superoxide, in the metal-catalyzed Haber–Weiss reaction can result in the production of the hydroxyl radical [8].

In this study, the antioxidants vitamin E, idebenone and GSH were tested, in order to gain a better insight into the mechanisms by which free radicals, formed after treatment with ascorbate/iron, induce lipid and protein alterations. Vitamin E is a lipophilic antioxidant that acts as a free radical scavenger, being able to donate hydrogen to radicals, usually peroxyl and alkoxyl radicals, thus preventing the propagation of lipid peroxidation [26]. Because it is localized into the hydrocarbon core of the phospholipid bilayer, it protects polyunsaturated fatty acids and thiol groups of membrane proteins, keeping the integrity and function of biomembranes [28]. Idebenone is also a lipophilic antioxidant, that besides acting as a free radical scavenger it can prevent free radical formation being an electron carrier, like ubiquinone [29]. The antioxidant GSH is involved in the inactivation of H_2O_2, being the substrate of glutathione peroxidase, it also acts as a free radical scavenger by giving H^+ to superoxide and hydroxyl radical [30] and prevents *s*-thiolation of proteins.

When synaptosomes are treated with ascorbate/iron, lipid peroxidation occurs (Table 1), as measured by the formation of free malonaldehyde (MDA), one of the last products formed during the peroxidative process [31]. Evidence exists that one of the major physiological targets of free radicals is the unsaturated fatty acyl side chains of phospholipids and their degradation leads to alterations of membrane fluidity and lipid composition [5,32,33]. An increase in LDH leakage was observed (Fig. 1) in synaptosomes treated with ascorbate/iron, suggesting that plasma membrane is functionally and structurally altered after membrane lipid peroxidation, as previously shown in our laboratory [34]. Vitamin E and idebenone were able to prevent the LDH leakage, by acting in the prevention of plasma membrane lipid peroxidation (Table 1). GSH also prevented the LDH leakage to a lesser extent. Besides the alteration in synaptosomal plasma membrane structure, mitochondria structural integrity is preserved, as can be observed by electron microscopy (Fig. 2).

There is considerable evidence that oxidative stress induces alterations not only in membrane lipids, but also in proteins and DNA [3,9]. Recently, Goldstein et al. [35] suggested that ˙OH radicals damage peptides in the presence of transition metal ions. After incubation of cortical synaptosomes with ascorbate/iron for 15 min, a significant decrease in the activity of the mitochondrial respiratory chain complexes II, III and V was observed (Table 2). In contrast, the activity of complex I and IV was not significantly affected upon oxidative stress induced by ascorbate and iron (Table 2). ATP levels, determined under these conditions, were also significantly decreased (Fig. 3), but ADP and AMP levels were maintained (data not shown). However, when synapto-

somes were incubated in the presence of idebenone, reduced glutathione (GSH), or in the presence of the three antioxidants (vitamin E, idebenone and GSH), ATP levels were similar to those of controls (Fig. 3). GSH prevented the drop in ATP levels but did not completely prevent LDH leakage (Fig. 1). Previous studies in our laboratory showed that the membrane potential of synaptosomes, treated with ascorbate/iron during 15 min, was similar to that of controls, suggesting that, under these conditions, synaptosomal plasma membrane Na^+/K^+ gradients are not significantly altered [36,37].

Oxidative stress has been shown to affect the activity of key mitochondrial enzymes, subsequently leading to a decline in ATP production [25,38,39]. Our results suggest that the maintenance of ATP levels is probably due to a protective effect of antioxidants on mitochondrial respiratory chain complexes, since vitamin E and idebenone, as well as the reducing agent GSH, were able to prevent the inhibition of complex III activity, while only GSH protected complexes II and V from the inhibitory effect induced by oxidative stress.

After the induction of oxidative stress with ascorbate/iron, Reinheckel [40] showed a decrease in complex I activity with a posterior inhibition of complex III activity and they proved that the decline of complex III activity, during the initial phase of lipid peroxidation, could be correlated to structural breakdown of the complex proteins. Andreé et al. [41] demonstrated that the depletion of the ubiquinone pool played an important role in the inhibition of the electron transfer chain (ETC), when lipid peroxidation occurred. They demonstrated that the preferential targets leading to the inhibition of the respiratory chain complexes I and II activities, were the partial reactions that involve ubiquinones. It has been shown that, under lipid peroxidation conditions superoxide radical, $O_2^{\bullet-}$, is released, because ubiquinones and cytochrome b family loose the capacity to retain the partially reduced oxygen intermediates, until oxygen is completely reduced to water [1,42]. The reaction of superoxide anion with Fe^{2+} can generate hydroxyl radicals that increase the hydrophilic properties of mitochondrial inner membrane lipids favoring the auto-oxidation of ubisemiquinones, leading also to an increased release of electrons from the normal chain sequence [43]. On the other hand, the ubisemiquinone formed can function as a reductant for hydrogen peroxide, leading to the formation of hydroxyl radicals [44]. However, this mechanism involving lipid peroxidation can not explain per se the decrease of complex II activity in synaptosomes treated with ascorbate/iron, because vitamin E and idebenone, that are lipid-soluble antioxidants inhibited lipid peroxidation without protecting complex II enzymatic activity. Our results show an inhibition of complex II activity, probably due to a direct damage on the

complex proteins, because this inhibition is prevented by reduced glutathione but not by vitamin E or idebenone (Fig. 4A). GSH, acting as a free radical scavenger, can help to regulate the thiol disulfide concentration of complex II subunits, as has been demonstrated for a number of glycolytic enzymes [9] and Ca^{2+}-ATPases [45]. Under severe oxidative stress reduced glutathione can be used as an antioxidant defense, and oxidized glutathione concentration increases. We found that in synaptosomes treated with ascorbate/iron, the GSH/GSSG ratio decreases (data not shown), suggesting an accumulation of GSSG and a possible oxidation of protein thiol groups, resulting in a possible alteration of mitochondria membranes and enzymes activity [46].

It has been reported that oxidative stress, either induced by oxidizing agents or occurring during aging, inhibits mitochondrial respiratory chain complexes (complex I and/or complex IV) [47–51]. However, the unchanged activities of complexes II, III and V, under peroxidative conditions, are far from being clarified. Studies with MPTP [48,50] revealed an inhibition of complex I activity, due to a free radical damage. The binding of MPP^+ to a specific site in complex I has been shown to induce ATP depletion and, as a consequence, leads to an impairment of GSH synthesis and GSH depletion. However, Gerlach et al. [52] showed that mitochondrial respiratory chain enzymes were not affected after treatment with MPTP, concluding that there was no evidence that the damage of complex I was the cause of the MPTP-toxicity. Bowling et al. [47] examined the activities of complex I, II/III, IV and V in aged brains, finding an age-related decline in complexes I and IV activities. These authors proposed that the inhibition of mitochondrial respiratory chain enzymes was due to a damage of mitochondrial DNA, that encodes for some of the complex I and IV subunits. Although complex III and V are also partially encoded by mitochondrial DNA [49], their activity has not been shown to be affected by age. More recently, Bolaños et al. [53] showed that complexes I, II/III and IV activities decreased in neurons exposed to the NO• donor, SNAP. This effect seems to be likely due to peroxynitrite, formed through the reaction of •NO with the superoxide anion. It has been reported that peroxynitrite can react with intracellular GSH, giving rise to cellular depletion of the antioxidant and mitochondria damage [53,54].

We also demonstrate a decrease in complex III activity (Fig. 4B), probably related to the production of superoxide radicals at the level of cytochrome b population, which has been shown to promote mitochondrial membrane alterations through the dismutase reaction in which hydrogen peroxide is formed. This effect is likely to be related to a direct damage of a hydrophobic component of the enzyme, since the decrease of complex III

activity was prevented by the free radical scavengers, vitamin E and idebenone (Fig. 4B). Vitamin E is one of the major lipid-soluble chain-breaking antioxidants in biological systems [26] that incorporate into the plasma membrane. It acts by interfering with the propagation of lipid peroxidation chain reaction [55], by scavenging lipid peroxyl and alkoxyl radicals [56]. Idebenone is a structural analog of ubiquinone [29], which has been suggested to act as an electron carrier, like ubiquinone and also as a free radical scavenger. Fry and Green [57] showed that the catalytic activity of complexes I and III of the mitochondrial respiratory chain needs a stable lipid environment. Cardiolipin is a negatively charged phospholipid required for the correct interaction of cytochrome c with Cytochrome Oxidase which can be decreased in the mitochondrial inner membrane after induction of oxidative stress [58]. However, under our experimental conditions, the peroxidation of cardiolipine does not seem to be involved in the inhibitory effect observed on complex III activity, since complex I and IV activities were unchanged, under the same conditions.

The decrease of ATP-synthase activity, after incubation with ascorbate/iron, was prevented only by GSH (Fig. 4C), indicating that this inhibition probably results from a direct attack of ROS on the complex proteins. Under these conditions, mitochondrial respiratory chain complexes proteins are oxidized, as is demonstrated by the increase in carbonyl groups formation (Fig. 5). These results are in agreement with other studies in which is predicted that protein oxidation can be acutely induced by the addition of Fe^{2+} [59]. When synaptosomes were pre-incubated with vitamin E, idebenone and GSH the increase in carbonyl groups levels was not observed, showing that membrane proteins are protected from free radicals attack as long as antioxidants do not decline. Our results suggest that the inhibition of mitochondrial activity mediated by oxidative stress can be related to the deficient regeneration of endogenous antioxidants, namely GSH that depends on the supply of hydrogen from respiratory substrates [50].

In conclusion, mitochondrial respiratory chain enzymatic complexes activity is differentially affected by oxidative stress, complex I and IV being the less vulnerable. The protection of complexes II, III and V activities, by free radical scavengers, suggests that the irreversible inhibition of these complexes, induced by oxidative stress, is mediated by free radical damage to lipids, but protein damage is also involved, as demonstrated by the GSH protective effect. This inhibition could lead to the generation of ROS by the mitochondria, thus enhancing the damaging events initiated by ascorbate/iron.

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ABBREVIATIONS

CoQ$_1$—ubiquinone 1
CoQ$_2$—ubiquinone 2
Cx I-NADH—ubiquinone oxidoreductase
Cx II—succinate-ubiquinone oxidoreductase
Cx II/III—succinate cytochrome c oxidoreductase
Cx III—ubiquinol cytochrome c reductase
Cx IV—cytochrome c oxidase
Cx V—ATP-synthase
DCPIP—2,6-dichlorophenolindophenol
DTNB—5,5′-dithiobis(2-nitrobenzoic acid)
LDH—lactate dehydrogenase
NO—nitric oxide radical
MPP$^+$—*1*-methyl-4-phenylpyridinium ion
MPTP—*1*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
SNAP—s-nitroso-*N*-acetylpenicillamine
TBARS—thiobarbituric acid-reactive substances
TTFA—thenoyltrifluoroacetone
UQ$_2$H$_2$—ubiquinol 2