INFLUENCE OF LIPID PEROXIDATION ON DOPAMINE RECEPTORS

A. P. Simões and C. R. Oliveira

Center for Cell Biology and Neurological Clinic
Universidade de Coimbra
3049 COIMBRA Codex, Portugal

ABSTRACT

The relationship between the binding activity of dopamine receptor in caudate nucleus microsomal fraction of sheep brain and lipid peroxidation of these membranes was examined. Caudate nucleus microsomal membranes were incubated with various concentrations of ascorbic acid in a Tris-HCl buffer. Ascorbic acid caused a decrease in ^{3}H-spiperone specific binding to caudate nucleus membranes and a parallel increase on membrane lipid peroxidation as measured by malonyldialdeyde (MDA) production. Both effects were greater at intermediate (1 mM) than at lower or higher ascorbic acid concentrations. This effect of ascorbic acid is time and temperature dependent and is inhibited by divalent cations, Ca^{2+}, Mg^{2+} and Mn^{2+} at 8 mM concentration. A reduction in the maximal number of ^{3}H-spiperone binding sites (Bmax decreases from 225 to 176 fmol/mg protein) and in the affinity (K_D increases from 0.21 nM to 0.30 nM) is observed after ascorbic acid induced membrane lipid peroxidation. An increase in the IC_{50} for spiperone, haloperidol, apomorphine and dopamine and a loss of stereospecific binding were also found. These results suggest that membrane lipids, sensitive to ascorbate induced peroxidation,play a critical role in the binding function of the receptor.

Key words: aging-lipid peroxidation - ascorbic acid - dopamine receptors

INTRODUCTION

Lipid peroxidation has been defined as an oxidative degradation of polyunsaturated fatty acids and it has been implicated in various pathological processes and in the physiological process of aging(1).

Lipid peroxidation usually begins with the abstraction of a hydrogen atom from unsaturated fatty acids, resulting in the formation of a lipid radical (2). The propagation of free radical chain reactions during membrane lipids peroxidation alters the physico-chemical properties of the membranes. However membrane proteins, that are involved in a wide range of functions, can also be damaged during the process (1).

The high content of polyunsaturated fatty acids in brain membranes makes the central nervous system particularly susceptible to lipid peroxidation (2). Recently it has been observed that ascorbate *in vitro* decreases the binding of radioligands to neurotransmitter receptors and it has been proposed that the ascorbate induced loss of receptor binding activity may result from the peroxidation of membrane lipids (3,4).

As a disfunction of the central nervous system occurs during the aging process and membrane lipid peroxidation has been implicated as one of the mechanisms through which cellular damage occurs, it seems of interest to investigate the role of membrane lipid peroxidation in senescence. Several age-related alterations in the dopaminergic system are apparently responsable for the disturbances associated with senescence (5).

Therefore we decided to investigate the effects of ascorbate-induced lipid peroxidation on dopamine receptor binding activity.

MATERIALS AND METHODS

Reagents: ^{3}H-spiperone (sp act. 19 Ci/mmol) was obtained from the Radiochemical Center (Amersham, U.K.); (±)butaclamol was kindly donated by Ayerst Research Laboratories (Canada). Spiperone and haloperidol were obtained from Janssen Pharmaceutica (Belgium); dopamine, apomorphine and thiobarbituric acid were obtained from Sigma (St Louis M.O.). All other chemicals were of reagent grade.

Preparation of microsomal fraction

Sheep brains were obtained fresh and caudate nuclei were removed within 1 hr after death and fractionated by the method described by Hajós (6). Caudate nuclei were sliced into small pieces and added to cold sucrose 0.3 M (1 gr w/w:9 ml sucrose) and were homogeneized in a glass homogeneizer with a Teflon piston. The supernatants obtained after centrifugation at 1 500xg for 10 minutes were centrifuged at 9 000xg for 20 minutes. The supernatants obtained were collected and centrifuged in a B-60 centrifuge at 39 000xg for 45 minutes at 4° C. The pellet was resuspended in Tris-HCl 15 mM, nialamide 12.5 µM (TN buffer) pH 7.4, at 4° C and washed twice with the same buffer. The final pellet was resuspended in a volume of TN buffer to obtain a protein concentration of 6 mg/ml, as determined by the biuret method (7). The microsomal fraction was stored in 2 ml aliquots, at -80° C for future use.

Lipid peroxidation

Ascorbic acid was used to induce lipid peroxidation (8) and the thiobarbituric acid (TBA) test was used to determine the extent of lipid peroxidation (9).

Ascorbic acid (0.6 mM) was added to 1 ml of the microsomal fraction (0.5 mg/ml of protein).

In some experiments ascorbic acid was used in 1 mM concentration and divalent cations were added just prior to the ascorbic acid. Samples, in a final volume of 2 ml, were then incubated at 37° C for 15 minutes. The reaction was stopped by lowering the temperature to 0-4° C by kepping the tubes on ice. Then, 0.5 ml of cold 50% trichloroacetic acid (w/v TCA) and 2 ml of 0.67% TBA were added to 0.5 ml of sample material and the tubes were treated for 10 minutes in a boilling water-bath using a marble as a condenser. The tubes were allowed to cool at room temperature and centrifuged at 3 000 rpm for 10 minutes. The supernatant was collected and the absorbance was read at 532 nm in a Spectronic 700 spectrophotometer. The amount of lipid peroxide was calculated by using a molar extintion coefficient of 1.56×10^5 $cm^2.min^{-1}$ and expressed as nmol MDA/mg protein as described previously (8).

^{3}H-spiperone binding assay

^{3}H-spiperone binding to microsomal membranes was measured in an incubation mixture (final volume of 1.8 ml) containing 0.6 ml of membrane preparation (0.3 mg of protein), 0.6 ml of ^{3}H-spiperone (0.25 nM final concentration) and 0.6 ml of TN buffer pH 7.4 (10). To define specific and stereospecific binding, (+) or (-)butaclamol (10^{-6} M final concentration) was added. The samples were incubated for 45 minutes at 25° C and the reaction was terminated by rapid filtration under vacuum of 0.5 ml aliquots through glass fiber filters (Whatman GF/B). The filter was washed with 10 ml of TN buffer at room temperature. Radioactivity in the filters was counted by liquid scintillation spectrometry in a Packard Scintillation Counter, Model 450C, corrected for quenching, with an efficiency of 62%, after overnight equilibration in 8.0 ml Triton X-100 Scintillation fluid (10). Specific and stereospecific binding were defined as the difference between the amount of ^{3}H-spiperone bound in the absence or in the presence of (-)-butaclamol and that in the presence (nonspecific binding) of 10^{-6} M (+)-butaclamol. The amount of ^{3}H-spiperone bound was expressed as fmol of ^{3}H-spiperone bound/mg of protein. All the assays were done in triplicate and the results were replicate at least three times in independently performed experiments. In some experiments, ^{3}H-spiperone (0.25 nM) was incubated in the presence of increasing concentrations of cold competing drugs (10^{-10} to 10^{-5} M).

The binding of ^{3}H-spiperone to microsomal membranes after membrane lipid peroxidation was compared with control samples. When membrane samples were incubated with ascorbic acid they were washed twice with TN buffer before ^{3}H-spiperone binding assay.

RESULTS

Characterization of ascorbic acid induced membrane lipid peroxidation

The effect of ascorbic acid concentration on the extent of membrane lipid peroxidation was examined (Fig. 1). Ascorbic acid, when incubated with the microsomal membranes at increasing concentrations (from 0.06 mM to 8.0 mM), induces an extensive lipid peroxidation with a maximum effect at 1 mM. There was less lipid peroxide formed at lower (0.06 mM) and at higher (6.0 mM) concentrations of ascorbic acid, as can be observed by the amount of MDA formed at these extreme ascorbic acid concentrations, 1.3 nmol/mg protein and 0.25 nmol/mg protein, respectively. In the absence of ascorbic acid no production of MDA was detected and the maximal amount of MDA formed was 3,12 nmol/mg protein in the presence of 1 mM ascorbic acid.

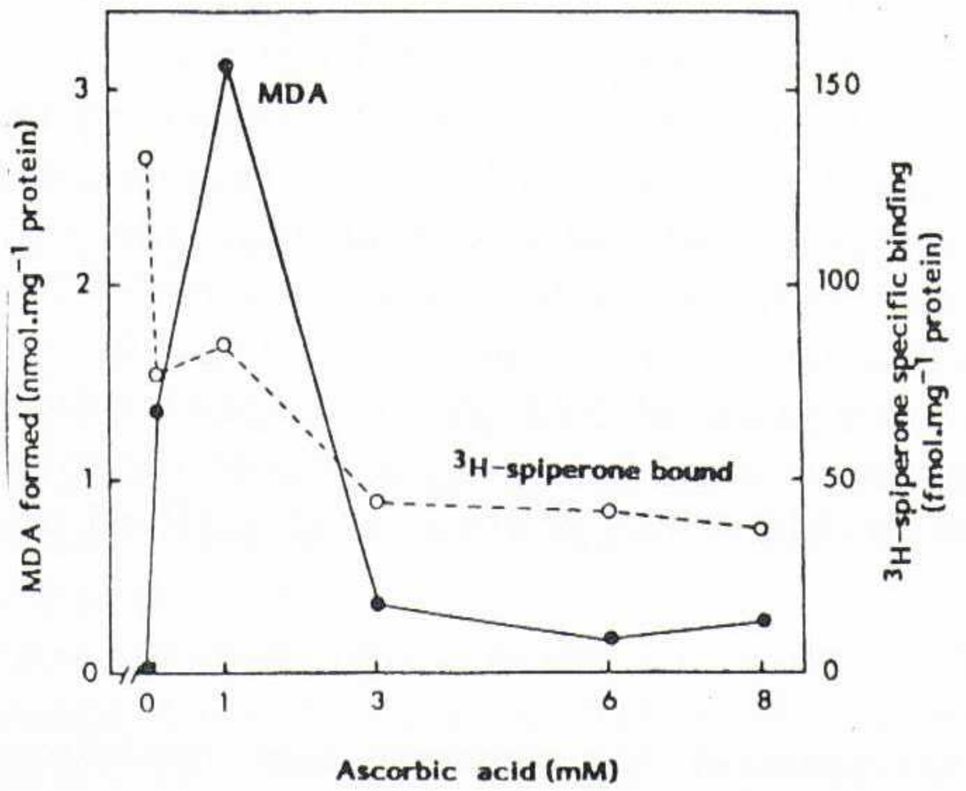

FIGURE 1

Effect of ascorbate concentration on MDA production in caudate nucleus microsomal fraction and on ^{3}H-spirerone binding to caudate nucleus microsomal fraction.
The experimental conditions were those described under Materials and Methods. The ascorbic acid concentrations changed from 0.06 to 6 mM. Data are expressed in nmol MDA formed per mg of protein and on fmol of ^{3}H-spiperone bound per mg of protein, respectively.

As can be observed in Fig. 2 and Fig. 3, membrane lipid peroxidation is a temperature and time-dependent process. The amount of MDA formed increase linearly as the incubation temperature increases (Fig. 2). The rate of production of MDA was aproximately linear in the first 15 minutes and then leveled of (Fig. 3).

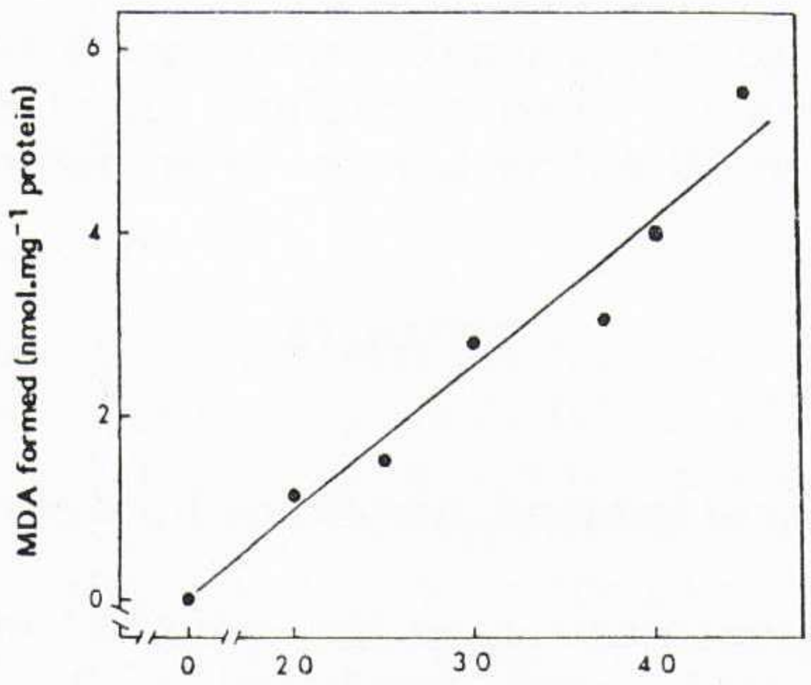

FIGURE 2

Effect of temperature on MDA production in caudate microsomal fraction.
The caudate nucleus microsomal membranes were incubated with ascorbic acid (1mM), for 15 minutes at different temperatures. The extent of membrane lipid peroxidation was measured by the amount of MDA formed at each incubation temperature, as described in Materials and Methods. The values are means of three experiments, each one run in triplicate.

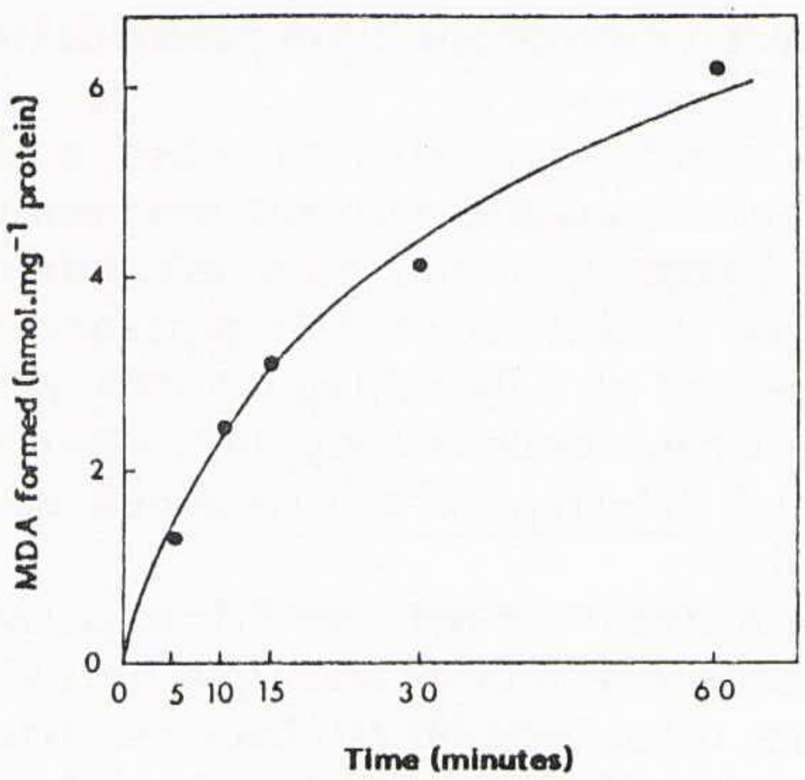

FIGURE 3

Effect of incubation time on MDA production in caudate nucleus microsomal fraction.
The microsomal membranes (0.5 mg/ml of protein) were incubated with 1 mM ascorbic acid at 37°C as described under Materials and Methods. The reaction was terminated at convenient intervals up to 1 hour and assayed for MDA produced at different incubation times. The values are means of three experiments each one run in triplicate.

The ascorbic acid membrane lipid peroxidation can be prevented by divalent cations (Table I). The large increase in lipid peroxide formation induced by 1 mM ascorbic acid (3.12 nmol/mg protein) can be prevented by Ca^{2+}, Mg^{2+} and Mn^{2+}, in a concentration range from 4 to 20 mM. Mn^{2+} is the most effective cation in this preventing effect, since at a 4 mM concentration it protects completely against the ascorbic-acid induced formation of lipid peroxide.

TABLE I

EFFECT OF $Ca^{2+}Mg^{2+}$ and Mn^{2+} ON MDA PRODUCTION BY CAUDATE MICROSOMAL FRACTION

Cations (mM)	Ca^{2+}	Mg^{2+}	Mn^{2+}
	MDA (nmol.mg^{-1} protein)		
0	2.35	2.13	2.13
4	1.35	2.07	——
8	1.7	1.3	——
10	1.06	0.78	——
12	1.06	0.78	——
16	0.78	0.81	——
20	0.64	0.54	——

The experimental conditions are those described under Methods, except for the presence of divalent cations. Each value represents the mean of three experiments each one run in duplicate.

Influence of ascorbic-acid-induced membrane lipid peroxidation on ^{3}H-spiperone binding

The effect of incubating the membranes with ascorbic acid on ^{3}H-spiperone binding was examined (Fig. 1). The incubation of caudate nucleus microsomal membranes with increasing concentrations of ascorbic acid (from 0.06 mM to 8 mM) shows a decrease in ^{3}H-spiperone specific binding. A very extensive inhibition of ^{3}H-spiperone binding at 1 mM ascorbic acid occurs and lesser effects are observed at 0.06 mM and 6 mM ascorbic acid concentrations.

The specific binding of ^{3}H-spiperone decrease by 46% when 3.12 nmol MDA/mg protein are produced. The specific binding of ^{3}H-spiperone to washed ascorbate-pretreated membranes is lower then in incubated controls.

Fig. 4A and B shows the saturation curves for ^{3}H-spiperone binding to caudate nucleus microsomal membranes after and before membrane lipid peroxidation, respectively. A marked inhibitory effect on ^{3}H-spiperone total binding is observed after peroxidation (Fig. 4A). Since no significant change on non-specific binding occurs, the inhibition of total binding is due to a decrease in specific binding.

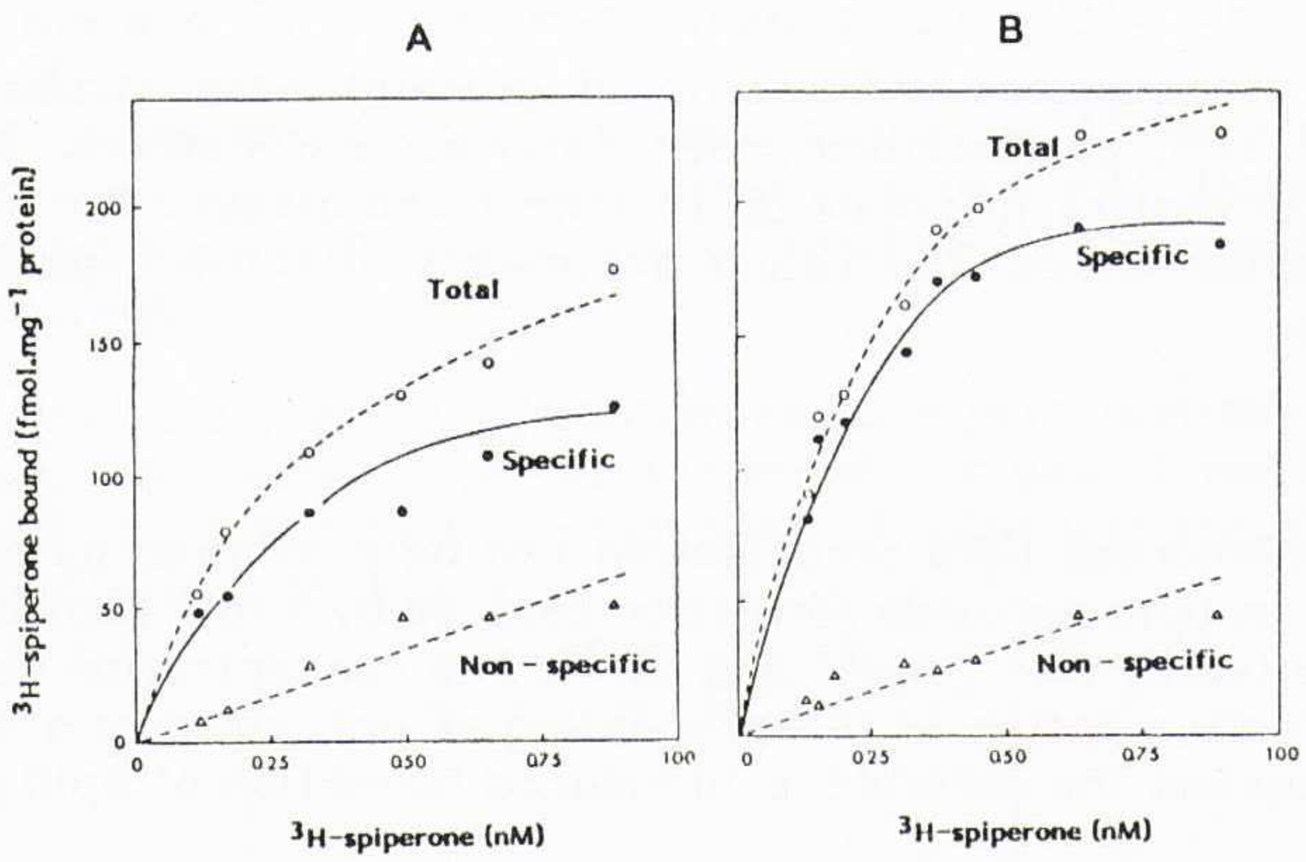

FIGURE 4

Saturation curves of ^{3}H-spiperone binding to caudate nucleus microsomal fraction.
Microsomal membranes were incubated with 1 mM ascorbic acid, at 37° C for 15 minutes as described under Methods. The washed ascorbate pretreated membranes (A) and incubated control membranes (B) were assayed for ^{3}H-spiperone binding at concentrations from 0.12 nM to 2 nM in the presence and in the absence of 1 µM (+)-butaclamol. The points on the figure are means of triplicate of three experiments. Control membranes signifies membranes submitted to the procedure above described but in the absence of ascorbic acid.

The scatchard analysis of these data (fig. 5) shows that a decrease in Bmax and K_D occurs after membrane lipid peroxidation. The maximal number of ^{3}H-spiperone binding sites decreases from 225 ± 9.4 fmol/mg protein in incubated control membranes to 176 ±6.8fmol/mg protein in ascorbic acid pre-treated membranes. The values for K_D change from 0.21 ± 0.042 nM to 0.30±0.014 nM respectively.

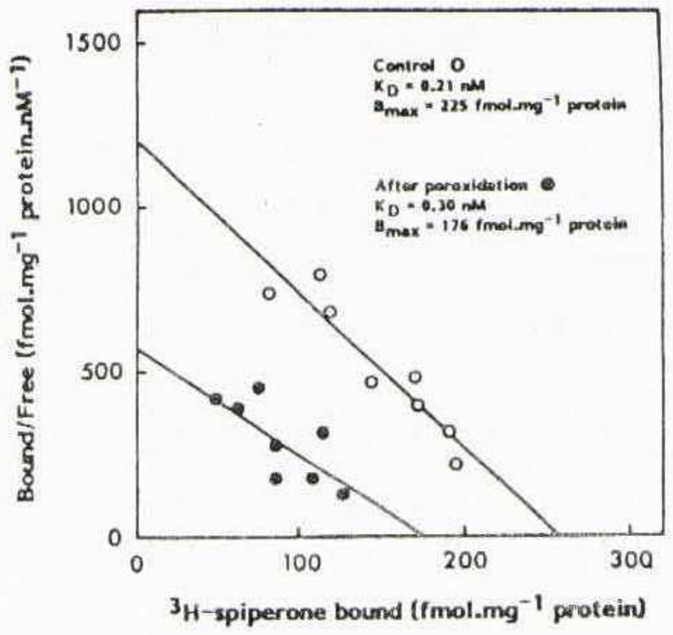

FIGURE 5

Scatchard plot of ^{3}H-spiperone specific binding to ascorbic acid pretreated membranes and to control membranes.

Caudate nucleus microsomal membranes were incubated with ascorbic acid in the experimental conditions refered in Fig. 4. Control membranes were incubated in the same conditions except for ascorbic acid addition. After incubation the membranes were sedimented and washed and assayed for ^{3}H-spiperone binding at concentrations from 0.12 nM to 2 nM in the presence and in the absence of 1 µM (+)-butaclamol.

All points in the figure are means of triplicate determinations from three experiments. Values calculated for K_D= 0.30±0.014 nM and for Bmax = 176 ±6.8 fmol/mg of protein in ascorbic acid treated membranes (●-●) are compared to the control values of K_D = 0.21±0.042 nM and Bmax = 225±9.4 fmol/mg of protein (o-o).

Really a decrease in^{3}H-spiperone stereospecific binding was observed after incubating the membranes with 1 mM ascorbic acid. The IC_{50} for ^{3}H-spiperone specific bonding calculated from competition curves with spiperone, haloperidol, dopamine and apomorphine, show a decrease in dopamine receptors affinity in peroxidated membranes (Table II). The Hill analysis of the data obtained from competition curves shows that there is no change in the Hill number after membrane lipid peroxidation (Table II).

TABLE II

IC_{50} VALUES AND HILL NUMBERS FOR SPIPERONE, HALOPERIDOL APOMORPHINE AND DOPAMINE BEFORE AND AFTER ASCORBIC ACID INDUCED LIPID PEROXIDATION

	Before Peroxidation		After Peroxidation	
Drugs	IC 50 (nM)	nH	IC 50 (nM)	nH
Spiperone	5.6	1.00	17	0.95
Haloperidol	17.7	0.97	70	0.88
Dopamine	15.8 x 10^3	0.60	500 x 10^3	0.40
Apomorphine	5 x 10^3	0.70	17.8 x 10^3	0.58

The membranes were incubated with ^{3}H-spiperone (0.25 nM) in the presence of increasing concentrations of cold competing drugs (10^{-10}M to 10^{-5}M). IC_{50} values and Hill numbers were calculated from competition curves. The values are means of three experiments each one run in triplicate.

DISCUSSION

The results of our study show that the incubation of caudate nucleus microsomal membranes with ascorbic acid has marked inhibitory effects on ^{3}H-spiperone binding to the membranes. The data also show that ascorbic acid causes an extensive lipid peroxidation as measured by MDA production. An "U-shapped" pattern of dose-response curve is observed on lipid peroxidation, ie: the extent of lipid peroxidation is higher at intermediate concentrations, with a maximum at 1 mM ascorbic acid, then at lower (0.06 mM) or higher (8 mM) concentrations. The specific binding of ^{3}H-spiperone in ascorbate-treated membranes is inversely correlated to the amount of MDA produced. With 8 mM ascorbic acid however, lipid peroxidation is almost completely inhibited, while some destruction of specific binding still occurs. The loss of ^{3}H-spiperone specific binding is observed even after washing of treated membranes what suggests that the blockage of the receptor binding site by lipid peroxide is not involved in the loss of specific binding.

Divalent cations, Ca^{2+} and Mg^{2+} inhibit ascorbic acid-induced lipid peroxidation and a complete inhibition is produced by Mn^{2+} even at the lowest concentration (4 mM). These cations have been shown to be potent inhibitors of the loss of spiperone specific binding in the presence of 1 mM ascorbic acid and Mn^{2+} was the most potent inhibitor of this destruction of specific binding (11). However this efect of Mn^{2+} on ^{3}Hspiperone specific binding can not be observed on the solubilized receptor (12). These observations are consistent with the hypothesis that one of the mechanisms involved in the ascorbic acid induced destruction of ^{3}H-spiperone specific binding is the peroxidation of membrane lipids induced by ascorbic acid.

Really, scatchard plots showed both a loss of ^{3}H-spiperone specific binding sites and a trend towards the decreased in ligand affinity for residual sites in ascorbate-treated membranes. A decrease in Bmax from 225 $\pm$ 9.4 fmol/mg protein to 176 $\pm$ 6.8 fmol/mg protein and an increase in K_D from 0.21 nM to 0.30 nM is observed after the treatment of caudate nucleus microsomal membranes by ascorbic acid . The increase in the IC_{50}values for dopamine agonists and antagonists after membrane lipid peroxidation is consistent with the decrease in dopamine receptor affinity. Both affinity states of the receptor seem to be equally sensitive to lipid peroxidation since the Hill numbers for agonists and antagonists do not change significantly after membrane lipid peroxidation.

The loss of spiperone binding in washed ascorbic-acid-treated membranes is due to a loss of the binding to the dopamine receptor since a decrease in ^{3}H-spiperone stereospecific binding is observed in treated membranes and membrane lipids do not display stereospecificity (10).

These results can be explained by the damage of both membrane lipids and proteins during the process of lipid peroxidation. Membrane lipid peroxidation has been shown to inhibit stereospecific binding to opiate receptors (13) and to β-adrenergic receptors (14). It has previously been reported that ascorbic acid can inhibit the binding of dopamine antagonists to striatal membranes by inducing membrane lipid peroxidation (4,15). However the mechanism by which these alterations in receptor binding activity are induced is not yet clarified. It is known that lipid peroxidation propagates itself through the hydrophobic core of the membrane and an increase in membrane viscosity has been detected after membrane lipid peroxidation (15,17). So it is not surprising that intrinsic membrane proteins may be affected during the process. On the other hand the products of lipid peroxidation can interact with membrane proteins. The citotoxic aldehydes that are formed during lipid peroxidation may interact with sulphydryl groups of the receptor protein, as has been observed in β-adrenergic receptors (13). Lipid peroxidation can also induce polimerization, clivage and denaturation of membrane proteins, as has been demonstrated in sarcoplasmic reticulum (18). Although the direct interaction of the products of lipid peroxidation with the receptor protein can not be ruled out,

it seems quite likely that the loss of stereospecific binding is not due to the blockage of the receptor binding site by lipid peroxide since washing of treated membranes removes lipid peroxide but does not restore stereospecific binding. Furthermore it has been shown before (12) that the binding of ^{3}H-spiperone to the solubilized receptor was not ascorbate sensitive. not ascorbate sensitive. All these data suggest that ascorbic acid reduces ^{3}H-spiperone stereospecific binding to dopamine receptors indirectly, by acting on the membrane lipids surrounding the receptor. We can conclude that, although the dopamine receptor is protein in nature, the lipid environement of the receptor is essential in maintaining the structural integrity of the receptor binding site, suggesting a role for both protein and lipid in the binding function of the receptor. This may have important implications in receptor disfunction observed not only in pathological states like dementia and cerebral ischemia but also in the phisiological process of aging where membrane lipid peroxidation has been implicated as a factor leading to irreversible functional injury of the cells.

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