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Autoxidation of Rat Brain Homogenate: Evidence for Spontaneous Lipid Peroxidation. Comparison with the Characteristics of Fe^{2+}- and Ascorbic Acid-Stimulated Lipid Peroxidation

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Aerobically-incubated brain homogenates are known to undergo autoxidation characterized by spontaneous TBARS production, presumably as a result of lipid peroxidation. However, TBARS measurement alone, because of its lack of specificity, is not sufficient to demonstrate the occurrence of lipid peroxidation in complex biological systems. This study, undertaken to determine whether or not spontaneous oxidation of rat brain homogenate is due to lipid peroxidation, measured different specific markers of this process (fatty acids, lipid aldehydes and the formation of fluorescence products) and studied changes in α-tocopherol.

Incubation of rat brain homogenates at 37°C under air led to spontaneous TBARS formation, which was accompanied by lipid aldehydes and lipid fluorescence products as well as polyunsaturated fatty acid (PUFA) degradation. Alpha-tocopherol was also consumed. On the whole, these results demonstrate that autoxidation of brain homogenate is a spontaneous lipid peroxidation process. When homogenates were exposed to Fe^{2+} and ascorbic acid-induced oxidative stress, lipid peroxidation was enhanced. However, spontaneous and stimulated peroxidation showed similar patterns not characteristic of classical lipid peroxidation, i.e. without the lag and accelerating phases typical of a propagating chain reaction. PUFA degradation was limited despite stimulation of peroxidation.

Keywords: Brain homogenate, autoxidation, lipid peroxidation, TBARS, Fe^{2+}–ascorbic acid-induced oxidation

Abbreviations: BHT, butylated hydroxytoluene; CHD, cyclohexanedione; DFO, desferrioxamine; GC–MS, gas chromatography–mass spectrometry; 4-HNE, 4-hydroxynonenal; HPLC, high performance liquid chromatography; MDA, malondialdehyde; PUFA, polyunsaturated fatty acid; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; TCA, trichloracetic acid; THF, tetrahydrofuran; TMP, 1,1,3,3-tetramethoxypropane

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INTRODUCTION

Oxygen free radicals have been implicated in the development of many neuronal disorders and brain dysfunction.[1–8] The brain appears to be particularly vulnerable to oxidative damage since it contains relatively high concentrations of readily peroxidizable fatty acids.[9,10] Various brain regions are highly enriched in iron,[11–15] which can catalyze the production of damaging oxygen free radical species. Moreover, the brain, which consumes a significant fraction of the total oxygen demand of the body,[16] is rather poorly endowed with protective antioxidant enzymes or antioxidant compounds.[17,18] Thus, the susceptibility of the brain to oxidative stress might account for an effect peculiar to this tissue *in vitro*: the autoxidation of brain homogenate expressed by spontaneous TBARS production during incubation in the absence of imposed stress. This effect was first described in 1944 by Kohn and Liversedge.[19] Subsequently, the autoxidation of brain homogenate, based on spontaneous TBARS production, was reported various times in the literature and very often attributed to spontaneous lipid peroxidation, since the terms "spontaneous autoxidation",[20,21] "spontaneous peroxidation",[22] "endogenous peroxidation"[15,23] and "basal lipid peroxidation"[24,25] have been used interchangeably. This property has also served for many years to measure the antioxidant activity of various substances.[20,22]

Although the TBARS test is currently used to assess lipid peroxidation, it has often been judged inadequate because of a lack of specificity.[26,27] Accordingly, the measurement of different classes of peroxidation products would seem necessary to confirm lipid peroxidation in complex biological material. Yet Matsuo *et al.*[18] recently found that the spontaneous TBARS production observed during incubation of brain homogenates was not correlated with other indices of lipid peroxidation and concluded that no lipid peroxidation occurs spontaneously when brain homogenates are incubated in the absence of a peroxidation initiator. These conflicting observations indicate that the origin of TBARS produced spontaneously during incubation of brain homogenates has not been clearly established.

To investigate whether or not spontaneous TBARS formation during incubation of brain homogenate is due to lipid peroxidation, the present study evaluated specific lipid peroxidation markers (fatty acids, lipid aldehydes, the formation of lipid fluorescence products) and the time-course of α-tocopherol, the major membrane antioxidant system. The changes in these markers were then compared with those obtained when homogenates were exposed to oxidative stress induced by Fe^{2+} and ascorbic acid.

MATERIALS AND METHODS

Chemicals

Methanol, chloroform, acetonitrile, tetrahydrofuran (THF), ethanol, *n*-hexane, benzene (all HPLC grade), ammonium sulphate and trichloracetic acid (TCA) 20% were purchased from Merck (Nogent sur Marne, France); *n*-hexanal (C6), 4-hydroxynonenal (4-HNE), cyclohexanedione (CHD), α-tocopherol, butylated hydroxytoluene (BHT), thiobarbituric acid (TBA), 1,1,3,3- tetramethoxypropane (TMP), pyrogallol, L-ascorbic acid and $FeCl_2 \cdot 4\ H_2O$ from Sigma (St. Quentin Fallavier, France); *n*-propanal from Aldrich (St. Quentin Fallavier, France); tocol from Hoffmann-LaRoche (Neuilly sur Seine, France); heptadecanoic acid (C17:0) from Interchim (Montluçon, France); and desferrioxamine (DFO) from Ciba-Geigy (Rueil-Malmaison, France).

Animals and Preparation of Brain Homogenates

Male Sprague–Dawley rats weighing 160–180 g (Dépré, St. Doulchard, France) were used in the experiments. The animals were anesthetized with

ether and perfused intracardially with cold saline solution in order to exclude effects of blood from intracranial vessels. After perfusion, brains were quickly removed from the skull and weighed. A 10% brain homogenate (w/v) was prepared in 0.05 M ice cold Tris-HCl buffer at pH 7.4, using a Teflon-glass homogenizer (8 up-and-down movements of the pestle revolving at 2,500 rpm, on ice).

Incubation conditions

Brain homogenates were incubated under an air atmosphere at 37°C in a shaking water bath with no addition, or after addition of 0.02 mM $FeCl_2$ and 0.25 mM ascorbic acid (final concentrations). At the end of the incubation period, DFO (final concentration 1 mM) and BHT (final concentration 0.2 mM) were added to stop any further lipid peroxidation.

Measurements

TBARS Determination

TBARS were measured according to the method of Wilbur *et al.*,[28] as modified by Sawas and Gilbert.[29] An aliquot (0.2 ml) of homogenate was brought to a volume of 1 ml with Tris-HCl buffer and mixed with 1 ml of 20% TCA. After centrifugation for 10 min at 3,000 rpm, 1 ml of the supernatant was added to 1 ml of an aqueous solution containing 0.67% TBA and heated at 100°C for 15 min. After cooling, samples were read on a spectrophotometer (Kontron, Uvikon 940) at 532 nm against a blank. The difference in absorbance between the sample and blank was used to calculate the TBARS concentration, employing an extinction coefficient of 156 $mmol^{-1}\,cm^{-1}$. Results are expressed as nmoles of TBARS produced per ml of homogenate.

Biochemical Markers of Lipid Peroxidation

Extraction and analysis of brain fatty acids: Brain lipids were extracted according to the procedure of Bligh and Dyer.[30] Aliquots (0.2 ml) of brain homogenate were transferred to a mixture of methanol : chloroform 2 : 1 (v/v). Heptadecanoic acid was added as an internal standard. The mixture was kept at +4°C for 1 h before centrifugation at 2,000 rpm. Four ml of the supernatant were added to chloroform (1.05 ml) and water (1.05 ml). The mixture was then kept at +4°C for 2 h. The phases were separated by centrifugation, and the chloroform phase (lower phase) was evaporated. The lipid extract was hydrolyzed with 1 N-NaOH in methanol : benzene (3 : 2 v/v) and transmethylated with 1 N-H_2SO_4 in methanol.

Fatty acid methyl esters were analyzed using a gas chromatograph (Girdel model 30, Delsi Instruments, France) equipped with a capillary column (25 m × 0.25 mm) packed with Carbowax 20 M (A.M.L.- Chromato, France). The oven temperature was programmed to rise from 170°C to 230°C at a rate of 2°C/min. The injector temperature was 230°C, and the detector temperature 270°C. Peaks were identified using a standard methylated fatty acid ester mixture on the basis of retention times, and measured with a programmable integrator-calculator (Shimadzu Chromatopac C-R1B). The concentrations of individual fatty acids (saturated fatty acids 16 : 0, 18 : 0 and polyunsaturated fatty acids 20 : 4, 22 : 6) were expressed as nmoles per ml of homogenate.

Lipid-soluble fluorescence material: These products were determined in the Bligh and Dyer extract, according to Bidlack and Tappel,[31] by spectrofluorometric assay. Fluorescence spectra were obtained with a Kontron SFM 25 spectrofluorometer. The high voltage selected was 320 V. Under these conditions, 1 μg of quinine sulphate/ml of 0.1 N H_2SO_4 had a relative fluorescence intensity of 100% (excitation 360 nm, emission 430 nm). The relative fluorescence intensities of the samples were expressed as μg of quinine sulphate per ml of homogenate.

α-Tocopherol determination: Alpha-tocopherol was determined by liquid separation and fluorimetric detection based on the method of Hatam

and Kayden[32] and Mino *et al.*[33] Briefly, 50 ng of tocol (internal standard) were added to 1 ml of brain homogenate. The mixture was then saponified at 70°C for 30 min after addition of 1 ml of 6% pyrogallol and 0.2 ml of 60% potassium hydroxide solutions. The tubes were cooled, and 2.5 ml of water was added, followed by 5 ml of hexane containing 0.1 ml of isoamyl alcohol. Tocopherol was extracted into the hexane phase by vigourous mixing for 3 min. The hexane phase was then separated out and evaporated down under a stream of nitrogen. The residue was redissolved in methanol, and 20 µl were analyzed by reverse-phase chromatography (microsphere C18, 5 µm, 4.6 × 100 mm, Chrompack, Les Ulis, France; mobile phase: methanol : water, 98 : 2; flow rate: 1.2 ml/min). Alpha-tocopherol was detected by its native fluorescence (excitation 285 nm, emission 340 nm).

Measurement of n-alkanals and 4-hydroxynonenal: Aldehydes (propanal, hexanal and 4-HNE) were determined according to the method of Holley *et al.*[34] Samples (0.25 ml homogenate brought to a volume of 0.5 ml with Tris-HCl buffer) were mixed with 1 ml cyclohexanedione (CHD) reagent [aqueous solution containing 0.25% CHD (w/v) and ammonium sulphate (10%, w/v)] and incubated at 60°C for 1 h. The mixture was then cooled on ice, and proteins were precipitated by addition of 0.5 ml methanol. Following centrifugation (10 min at 1,500 rpm), 1 ml of supernatant was applied to a Sep-Pak C_{18} cartridge (Waters, St. Quentin Yvelines, France) which had been preconditioned with 2 ml methanol followed by 4 ml water. The Sep-Pak was washed with 2 ml water, and the aldehyde–CHD derivatives were eluted with 2 ml methanol, after which 20 µl of eluate were analyzed by HPLC under the following conditions: column, Spherisorb ODS 2 (4.6 × 250 mm, Phase Sep, St. Quentin Yvelines, France); pump, Meck model L-6200; rheodyne injector model 7125 (20 µl sample loop); spectrophotometer, Shimadzu model RF-551 (excitation 380 nm, emission 445 nm). The elution system consisted of a linear gradient from 100% aqueous THF 20–40% aqueous THF 20%/60% acetonitrile over 30 min (flow rate, 0.7 ml/min). Standards were run in parallel with samples, and peak areas were used to determine sample aldehyde concentration (expressed as nmol aldehyde per ml of homogenate) after subtraction of reagent blank values.

MDA–TBA complex determination: The MDA–TBA complex was determined according to the methods of Lazzarino *et al.*[35] and Chirico.[36] Aliquots of homogenate (0.4 ml) were deproteinized by 0.6 M ice-cold $HClO_4$ (1 : 2, v/v) and neutralized by addition of 5 M K_2CO_3. To the extract (0.25 ml) or a 1,1,3,3-tetramethoxypropane (TMP) standard (prepared in ethanol/water 40 : 60, v/v) was added 0.75 ml of 0.44 M H_3PO_4. 0.25 ml of TBA solution (0.67%, w/v in distilled water) was then added, and the mixture was heated at 100°C for 45 min. After cooling on ice, 20 µl of the mixture were injected onto a 5 µm Lichrospher column 100 RP18 (4.6 × 250 mm, Merck) and eluted with 65% 50 mM KH_2PO_4/15% methanol/20% acetonitrile at a flow rate of 0.9 ml/min. The TBA–MDA adduct was detected by a UV/visible detector (Hewlett Packard, Series 1050) set at 532 nm. The identity of the peak was confirmed by spiking with the TBA–MDA adduct produced using a TMP standard. MDA–TBA complex was quantified from the calibration curve of TMP and the results were expressed as nmoles per ml of homogenate.

Ascorbic Acid Measurement

Ascorbic acid (reduced form) concentrations were determined according to the method of Lavigne *et al.*[37] using the supernatant of a 100,000 × *g* centrifugation of brain homogenate. Twenty microliters of the supernatant were directly injected into the HPLC system, which consisted of a Hypersil ODS (4.6 × 200 mm, Waters) column, a model 7125 rheodyne injector,

and a UV/visible detector (Hewlett Packard, Series 1050) set at 280 nm. The mobile phase consisted of 22 mM potassium phosphate buffer (pH = 6.45) containing one low-UV PIC B6 reagent vial in methanol pumped at a flow rate of 0.5 ml/min. L-ascorbic acid standards were run in parallel with samples. Peaks areas were used to determine ascorbic acid concentrations which were expressed as nmoles per ml of homogenate.

Statistical Analysis

The paired Student's *t*-test was used for statistical calculations ($p < 0.05$ was considered significant).

RESULTS

Spontaneous Oxidation of Rat Brain Homogenates

Figure 1 shows that incubation of brain homogenate at 37°C under air gives rise to spontaneous TBARS which increased immediately (without any lag phase) and rapidly during the first hours of incubation. A plateau value was reached after approximately 4 h of incubation. When incubation was performed under 100% nitrogen or in the presence of 0.2 mM BHT or 1 mM DFO, no TBARS accumulation was observed (< 0.5 nmol/ml of homogenate in all three incubation conditions). Moreover, Figure 1 shows that the kinetics of MDA production, as measured by the HPLC method, were closely correlated with that of TBARS (as measured by the colorimetric method).

To evaluate whether or not spontaneous TBARS formation was due to lipid peroxidation, more specific lipid peroxidation markers were investigated. Table I shows that spontaneous TBARS production was accompanied by a significant decrease in 20:4 and 22:6 fatty acids ($p < 0.01$ and $p < 0.005$ respectively) while there were no differences in the saturated fatty acids (16:0 and 18:0). When the incubation period was prolonged to 24 h, PUFA concentrations continued to decrease, although at a slower rate.

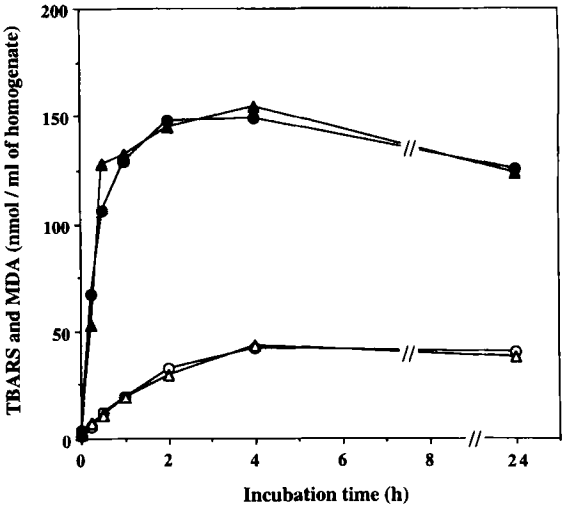

FIGURE 1 Time-course of TBARS (●/○) and MDA (▲/△) formation by rat brain homogenate after incubation either without (spontaneous) or with 0.02 mM Fe^{2+} and 0.25 mM ascorbic acid (stimulated lipid peroxidation). Incubation was performed at 37°C under air. Open symbols: without addition; filled symbols: with Fe^{2+}–ascorbic acid. For each incubation condition, the curves represent one of three separate experiments carried out with three different brains. For TBARS and MDA, the data are from the same experiments.

As shown in Figure 2A, propanal (formed from 22:6), hexanal and 4-hydroxynonenal (4-HNE), both formed from 20:4, were also produced spontaneously during 24-h incubation of brain homogenate. Although propanal and hexanal concentrations increased during the first 15 min of incubation, 4-HNE was only measurable after 1 h of incubation. Aldehyde concentrations increased steadily up to 4 h and then decreased.

The appearance of fluorescence material was determined in the lipid extract. Figure 3A shows the emission fluorescence spectrum for one of the three experiments conducted. A fluorescence peak characteristic of Schiff bases appeared in the lipid extract after 1 h of incubation. (E_X/E_M 360/430 nm). Fluorescence intensity increased with incubation time during a 24-h period. Table II relates the loss of each PUFA to the levels of corresponding aldehyde formed and to the increase in fluorescence intensity measured after 4 and 24 h of incubation.

TABLE I Changes in fatty acid concentrations of rat brain homogenates incubated without additions (spontaneous oxidation) or with 0.02 mM Fe^{2+} and 0.25 mM ascorbic acid

Incubation conditions	Incubation time	Fatty acid concentrations (nmol/ml of homogenate)			
		16:0	18:0	20:4	22:6
Without additions ($n=5$)	$t=0$	2293 ± 122	2285 ± 96	1213 ± 101	1600 ± 121
	$t=4$ h	2284 ± 143	2382 ± 197	1109 ± 70	1374 ± 121**
	$t=24$ h	2234 ± 47	2151 ± 103	925 ± 88**	1101 ± 172***
With Fe^{2+}–ascorbic acid ($n=4$)	$t=0$	2303 ± 84	2383 ± 190	1162 ± 92	1663 ± 139
	$t=4$ h	2118 ± 138**	2136 ± 239*	867 ± 101**	892 ± 38***
	$t=24$ h	2004 ± 164*	1812 ± 185***	662 ± 55***	618 ± 64***

Values are means ± SD of separate experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$ significantly different from $t = 0$ respectively.

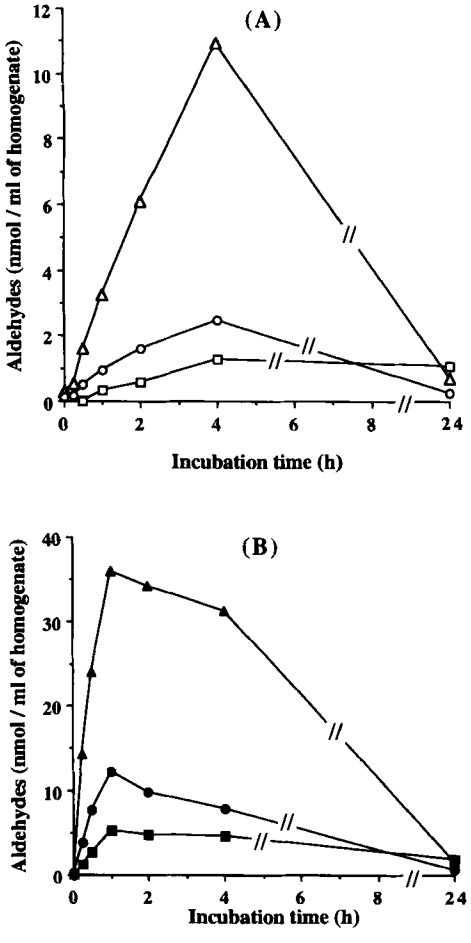

FIGURE 2 Time-course of propanal (▲/△), hexanal (●/○) and 4-hydroxynonenal (■/□) during 24-h incubation (A) without (spontaneously) (B) or with addition of 0.02 mM Fe^{2+} and 0.25 mM ascorbic acid. The curves show the results of one of three identical experiments with different brains.

Table III shows the results of α-tocopherol levels in brain homogenates after 4 and 24 h of incubation at 37°C. After 4 h, without addition of a peroxidative system, a highly significant decrease in α-tocopherol level was noted ($p < 0.001$), which continued with increasing incubation time. After 24 h of incubation, approximately 80% of the α-tocopherol had disappeared.

Comparison of Spontaneous Oxidation of Brain Homogenate with Fe^{2+}–Ascorbic Acid-Stimulated Lipid Peroxidation.

The data for the time-course of Fe^{2+}–ascorbic acid-induced TBARS formation in brain homogenate are given in Figure 1. The addition of 0.02 mM Fe^{2+} and 0.25 mM ascorbic acid greatly enhanced TBARS production in brain homogenate. The appearance of these substances was immediate and more rapid than in the absence of a peroxidant system, but their changes were parallel to those obtained spontaneously during incubation at 37°C. As in the previous experiments, MDA levels at each incubation time (as measured by the HPLC method) were superimposable with those for TBARS (Figure 1).

Fe^{2+}–ascorbic acid-induced peroxidation showed a greater decrease in the PUFA concentrations after 4-h incubation than in spontaneous oxidation conditions (Table I). This decrease was more important after 24 h of incubation, but not as great as that recorded during the first 4 h of incubation. After 24 h of incubation with Fe^{2+} and

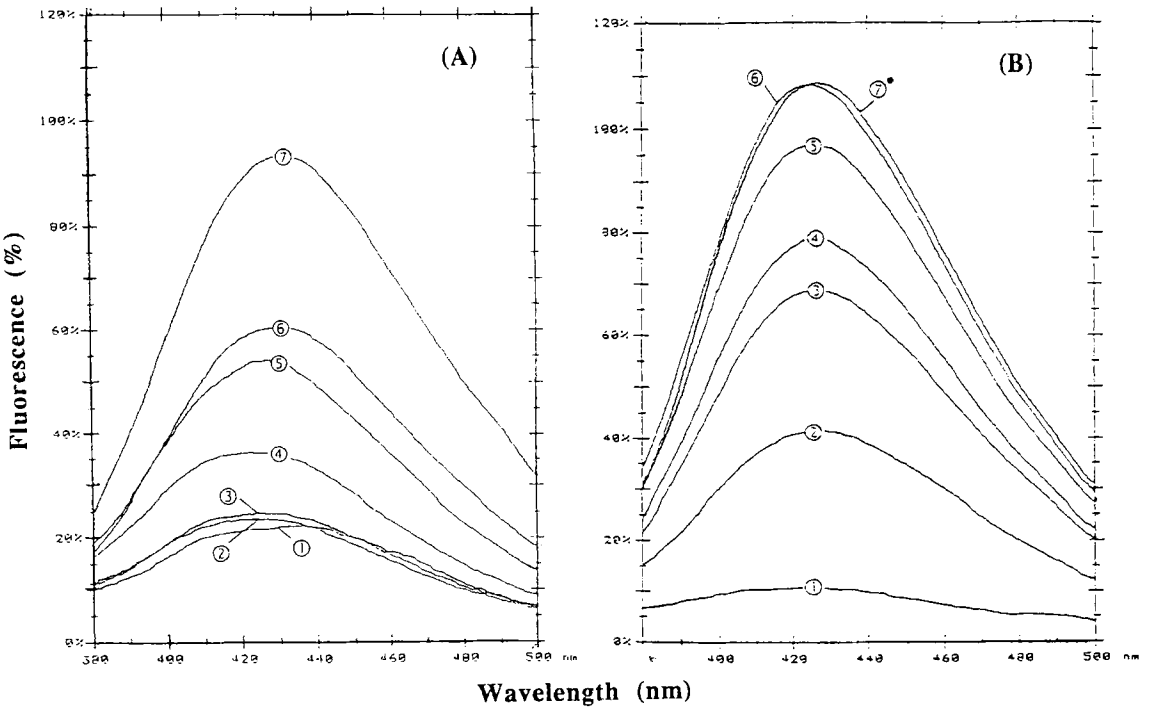

FIGURE 3 Emission fluorescence spectra of lipid extracts from brain homogenates (A) incubated without (B) or with 0.02 mM Fe^{2+} and 0.25 mM ascorbic acid. The figures show the results of one of three identical experiments with different brains. (1): before incubation (zero time sample); (2–7): incubation for 15 min, 30 min, 1 h, 2 h, 4 h and 24 h respectively. Lipid extracts were dissolved in 0.3 ml of chloroform : methanol (2 : 1, v/v) when incubation was performed without any additions, and in 0.5 ml (2 : 1, v/v) with Fe^{2+}–ascorbic acid-stimulated lipid peroxidation. (7)* Lipid extract was dissolved in 1 ml of chloroform : methanol (2 : 1, v/v).

TABLE II Loss of PUFAs, formation of aldehydes and fluorescent products during incubation of rat brain homogenates with and without 0.02 mM Fe^{2+} and 0.25 mM ascorbic acid. Data of PUFA loss and increase in fluorescence are given for 4 h and 24 h of incubation. Data of aldehyde production are given for 4 h of incubation

PUFA	Incubation without addition	Incubation with Fe^{2+}–ascorbic acid
20 : 4 loss (nmol/ml)	104/<u>288</u>	295/<u>500</u>
22 : 6 loss (nmol/ml)	226/<u>499</u>	771/<u>1045</u>
MDA production (nmol/ml)	48.4 ± 7.7	156.7 ± 27.3
Propanal[a] (nmol/ml)	12.6 ± 2.2	37.3 ± 7.9
Hexanal[b] (nmol/ml)	2.6 ± 0.3	9.1 ± 2.0
4-HNE[b] (nmol/ml)	1.3 ± 0.2	4.3 ± 0.8
Increase in fluorescence intensity (Quinine sulphate, μg/ml)	0.5 ± 0.1/<u>0.9 ± 0.3</u>	1.2 ± 0.2/<u>2.4 ± 0.4</u>

Values are means ± SD of 3–5 experiments performed on different brains. Data underlined represent values obtained after 24 h of incubation. [a]Formed from 22 : 6; [b]formed from 20 : 4.

ascorbic acid, 50% of 20 : 4 and 60% of 22 : 6 have disappeared. However, between 4 and 24 h of incubation, the loss of PUFAs was similar to that obtained during spontaneous oxidation (479 and 457 nmoles of PUFAs per ml of homogenate, respectively).

Figure 2B shows the appearance of aldehydes (propanal, hexanal and 4-HNE) resulting from

TABLE III Alpha-tocopherol levels in rat brain homogenates undergoing spontaneous oxidation or Fe^{2+}–ascorbic acid-stimulated lipid peroxidation (incubated for different time periods with or without 0.02 mM Fe^{2+} and 0.25 mM ascorbic acid) Results are given as the means ± SD of separate experiments

Incubation conditions	α-tocopherol levels (nmol/ml of homogenate) at incubation time, *t*		
	t = 0	*t* = 4 h	*t* = 24 h
Without additions (*n* = 5)	3.501 ± 0.649	1.574 ± 0.492 **	0.639 ± 0.105 *
With Fe^{2+}–ascorbic acid (*n* = 5)	3.501 ± 0.649	0.692 ± 0.188 **	0.318 ± 0.067 (*n* = 3)

* $p < 0.01$; ** $p < 0.001$ significantly different from $t = 0$ respectively.

PUFA degradation during the oxidative stress imposed by Fe^{2+} and ascorbic acid. Propanal and hexanal were present in large quantities from the first 15 min of incubation. 4-HNE, which was quantifiable only after 2 h when incubated alone, increased quite markedly after only 15 min of incubation in these conditions. Aldehydes increased very rapidly during the first hour of incubation and then decreased between 4 and 24 h of incubation.

Figure 3B shows that fluorescence of the lipid extract was observed from 15 min of incubation. Fluorescence intensity increased markedly with incubation time over the 24-h period and was much more intense than in the absence of imposed oxidative stress. As shown in Table II, the changes in fluorescence intensity were related to the PUFA decreases at the end of 24 h of incubation.

Table III shows that α-tocopherol levels decreased markedly during the first 4 h of incubation in the presence of Fe^{2+} and ascorbic acid. After 24 h of incubation, only 10% of the initial α-tocopherol level was present.

Since ascorbic acid is highly concentrated in brain and can behave like an oxidant or an antioxidant, we also examined the time-course of ascorbic acid during spontaneous and stimulated oxidation processes.

As shown in Figure 4A, endogenous ascorbic acid levels decreased spontaneously when brain homogenates were incubated aerobically at 37°C in the absence of any peroxidative system. This decrease occurred mainly during the first 4 h of incubation, concomitantly with an increase in TBARS values, and then remained at baseline level until 24 h of incubation. In the presence of Fe^{2+} and ascorbic acid (Figure 4B), the rate of degradation of ascorbic acid was greatly enhanced. After 1 h of incubation, 82% and 19% of the initial ascorbic acid concentrations remained for spontaneous and stimulated oxidation respectively.

It should be mentioned that a second Fe^{2+}–ascorbic acid-induced oxidative stress applied to the homogenate after 4 h of incubation (i.e. after the plateau was reached for TBARS) led to an instantaneous and rapid resurgence of TBARS production (data not shown). Moreover, the TBARS kinetics showed a similar profile to that observed during the first period of oxidative stress, proving that the development of the reaction depended on the efficiency of the oxidant system.

DISCUSSION

Our results show that aerobic incubation of brain homogenate at 37°C produced a spontaneous and immediate increase in TBARS. The fact that this production was inhibited by DFO demonstrates the role of iron in this process. Moreover, TBARS production was inhibited by BHT or when incubation was conducted under a nitrogen atmosphere, indicating that an oxidative process was involved which warrants the descriptive term "autoxidation." These results are consistent with those reported by others.[15,18,20,21,23] Yet, even though this phenomenon is known, no study to date has demonstrated that these TBARS products arise spontaneously from lipid peroxidation. For this reason, we studied changes in fatty acids, lipid aldehydes, the fluorescence of

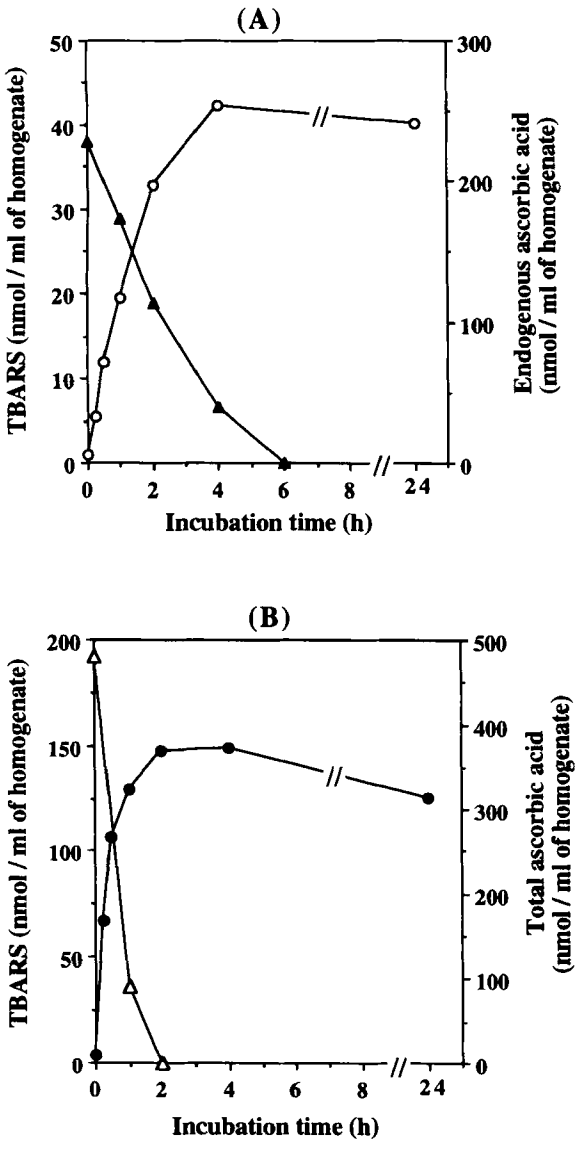

FIGURE 4 Time-course of (A) endogenous ascorbic acid (▲) during 24 h of incubation of rat brain homogenate at 37°C under air and (B) total ascorbic acid (△) during incubation with 0.02 mM Fe^{2+} and 0.25 mM ascorbic acid. The figure shows the results of one of three identical experiments with different brains. The time-course of TBARS formation is shown in parallel (●/○).

lipid extract and α-tocopherol, all of which are more specific variables.

Spontaneous TBARS production during aerobic incubation of brain homogenate at 37°C was accompanied by a selective loss of arachidonic and docosahexaenoic acids. The concomitant appearance of MDA, propanal, hexanal and 4-HNE, which were the major aldehydes generated during lipid peroxidation,[38] was indicative of PUFA degradation. The higher levels of propanal (formed from ω 3 PUFAs) in relation to those of hexanal and 4-HNE (both formed from ω 6 PUFAs) was in good agreement with what was expected since 22 : 6 fatty acid was predominantly degraded. However, these three aldehydes were detected in lower quantities than those of MDA. Two reasons might explain this result. First, MDA can be formed from both 20 : 4 and 22 : 6 fatty acids; in addition, a part of MDA is possibly formed from non lipid biomolecules.[26] Secondly, MDA is a poorly reactive species in comparison with others aldehydes.[15,38,39] Aldehydes constitute intermediate products in the reaction sequence which can react rapidly (especially 4-HNE) with -SH and $-NH_2$ residues. This would be consistent with the increase in fluorescence (E_X/E_M 360/430 nm) over 24 h of incubation, reflecting the accumulation of stable end-products of lipid peroxidation.

Taken as a whole, our results indicate that autoxidation of brain homogenate is a spontaneous lipid peroxidation process. However, this spontaneous lipid peroxidation showed particular characteristics since TBARS production was immediate without a lag phase. Since biological systems have antioxidants such as α-tocopherol, their lipid peroxidation *in vitro* can be expected to arise after an induction period during which antioxidants are consumed. However, in our conditions, TBARS production and the decrease in α-tocopherol were simultaneous. Moreover, PUFA degradation arose predominantly during the fourth hour of incubation. Thus, this process was not equivalent to a usually described lipid peroxidation, which is an auto-accelerating chain reaction that should be expected to lead to a greater degradation of PUFAs by the end of 24-h incubation. Instead, the reaction began instantaneously and did not appear to

develop or accelerate after the first hours of incubation.

In a second set of experiments, we compared the time-course of changes of the different parameters with that obtained during an Fe^{2+}–ascorbic acid-stimulated peroxidation. This system is used classically to stimulate lipid peroxidation *in vitro*.[15,24,40] As expected, the addition of iron and ascorbic acid intensified the effects observed spontaneously. TBARS, aldehydes from PUFA degradation and lipid fluorescence products increased much more rapidly and were produced in greater quantity. PUFA degradation and the consumption of α-tocopherol were also more marked. The changes in all these variables were parallel, whether a peroxidant system was added or not. However, despite stimulation of peroxidation, PUFA degradation proceeded slowly between 4 and 24 h of incubation, after the major attack during the first 4 h. A similar slowing of the reaction has been previously reported.[40,41] Thus, the effects of oxidative stress would appear to be limited in time. Lipid peroxidation of brain homogenate, whether stimulated or spontaneous, does not seem to develop or accelerate after the first hours of incubation.

These results reveal a paradox between spontaneous lipid peroxidation of the brain, indicative of the marked fragility of this tissue with respect to oxidative events, and its unexpected resistance to imposed oxidative stress, as evidenced by the limited PUFA degradation after 24 h of incubation.

The abundance of PUFAs in the brain, as compared to other tissues, and the presence of an endogenous oxidant system composed of iron [11,15] and ascorbic acid,[42] could account for spontaneous cerebral oxidation. However, what is the explanation for the slower rate of development of the reaction after several hours of incubation? One possibility is that the slowdown occurs once the oxidative system (particularly ascorbic acid) is consumed. Accordingly, we examined the time-course of endogenous and total ascorbic acid during the spontaneous oxidation process and Fe^{2+}–ascorbic acid-induced lipid peroxidation. Our results showed that endogenous ascorbic acid levels decreased at a similar rate to that of the TBARS increase during the 4 h of incubation. When Fe^{2+}–ascorbate was added to the homogenate, the rate of decrease of total ascorbic acid was faster than in the absence of the peroxidative system and paralleled the time-course of TBARS. Thus, ascorbic acid apparently fell to undetectable levels after the first hours of incubation and was thus unable to recycle iron from the ferric to the ferrous state. The reaction then proceeded more slowly since Fe^{3+} is less effective in stimulating free radical reaction than Fe^{2+}. This would be consistent with the identical loss of PUFAs observed between 4 and 24 h of incubation during both spontaneous and stimulated peroxidation and also with the resurgence of TBARS induced by the re-addition of Fe^{2+}–ascorbate after 4 h of incubation.

In conclusion, the changes in all the variables measured demonstrate that spontaneous oxidation of brain homogenates is an Fe^{2+}–ascorbic acid-dependent lipid peroxidation. Spontaneous and stimulated lipid peroxidation of brain homogenate shows the following characteristics: (i) despite the presence of α-tocopherol, the reaction began immediately without a lag-phase; (ii) the peroxidation developed rapidly until ascorbic acid disappeared, and then proceeded very slowly. This suggest that the changes in the reaction were related to the efficiency of the oxidative system. Thus, brain homogenate did not undergo a typical chain-reaction propagation of lipid peroxidation which should theoretically continue even if the initiator has been suppressed. Conversely, this peroxidation reaction was not self-sustained.

The fact that lipid peroxidation can develop spontaneously and instantaneously *in vitro* when brain is homogenized suggest, as proposed by Halliwell,[11] that *in vivo* "injury to brain by mechanical means (trauma) can lead to a partial disruption of brain tissue" ("partial homogenization") favorable to local peroxidation.

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