Vol. 145, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 29, 1987 Pages 211-217

THE EFFECT OF EDTA-Fe(III) COMPLEXES WITH DIFFERENT CHEMICAL STRUCTURE ON THE LIPID PEROXIDATION IN BRAIN MICROSOMES

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Received March 2, 1987

SUMMARY: Unlike EDTA-Fe(III) (1:1), the oxidized form of EDTA-Fe(II) complex enhanced lipid peroxidation in brain microsomes. Mossbauer spectroscopy and electron paramagnetic resonance analysis of oxidized EDTA-Fe(II), capable of inducing lipid peroxidation, showed the presence of EDTA-Fe(III) complex, which was different from the separately prepared (not oxidized) EDTA-Fe(III).
Lipid peroxidation initiated by the oxidized EDTA-Fe(II) complex was dependent on the presence of NAD(P)H and functionally intact microsomes. No inhibitory effect was found by generally used free radical scavengers and catalase.
Our results clearly indicate that the chemically different EDTA-Fe(III) complexes differ in their capability of initiating the NAD(P)H-dependent lipid peroxidation in brain microsomes. © 1987 Academic Press, Inc.

In recent years extensive studies were carried out concerning the mechanism of in vitro lipid peroxidation in various systems in order to understand the molecular mechanism of the process and the role of iron-complexes in the enhancement of initiation (1,2,3).
The supposed agent which could initiate lipid peroxidation was proposed to be one of the partially reduced oxygen species (4,5,6), a dioxygen iron complex (7,8,9), or iron reacting in a modified Fenton reaction directly with the traces of lipid hydroperoxide present (10,11).

Abbreviation used: EDTA, ethylenediamine tetraacetic acid; MDA, malondialdehyde; SOD, superoxide dismutase; EPR, electron paramagnetic resonance

0006-291X/87 $1.50
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We describe now an approach to this problem by using, as starting point our recent observation, that the iron complex induced, NAD(P)H dependent lipid peroxidation behaves differently in brain microsomes (12).
Further analysis of the effect of EDTA-iron complexes, would be presented here, proving that the initiation of lipid peroxidation requires a well determinated structure of the complex.

MATERIALS AND METHODS

Materials

All chemical materials used were obtained from Reanal Co (Budapest).

Preparation of brain microsomes

Microsomes were prepared from the brain of wistar rats weighing 150-200 g. Whole brains were homogenized (10 W/V%) in 0,25 M sucrose solution. The homogenate was centrifuged 15.000 x g for 20 min. The microsomes were pelleted by centrifugation at 78.000 x g for 60 min. from the supernatant. The pellet was washed once and suspended in 0.15 M KCl(10 mg/ml protein) then stored at $-20^{o}C$.
Protein estimation was performed according to Lowry et al. (13).

Determination of lipid peroxidation activity

Rat brain microsomes (0,5 mg/ml) were incubated at $37^{o}C$ in a shaking water bath under air; in the presence of 20 μM iron, in the form of ammonium-sulphate salt, 300 μM NAD(P)H and 20 μM EDTA in 50 mM tris-maleate buffer pH=6.8 (standard reaction mixture). Experiments were started by the addition of iron. Due to the quick autoxidation of EDTA-Fe(II), the fresh ferrous salt was not previously mixed with EDTA.
Aliquots from reaction mixtures were assayed for TBA reactive material by the procedure of Buege and Aust (14). The results were expressed as nmole MDA per mg protein using an extinction coefficient of $1.56 \cdot 10^{5} M^{-1} cm^{-1}$.

EPR and Mossbauer measurements

X-Band EPR measurements were carried out at $80^{o}K$ with an EPS spectrometer. The 50 mM water solutions of Fe(II) and Fe(III)-ammonium-sulphate were frozen rapidly in the EPR sample holder by liquid Nitrogen.
EDTA-Fe(III) aqueous solutions of 0.5 M concentration were prepared for Mossbauer model measurements.(The concentration, used for biochemical reactions, is too low for the Mossbauer experiments.) The solutions were quenched in liquid nitrogen and the Mossbauer spectra were recorded at $80^{o}K$ by a Ranger Electronics spectrometer. Lorentzian functions were used for the fitting of the spectra.

RESULTS and DISCUSSION

The effect of EDTA-iron complexes on the lipid peroxidation in brain microsomes.

In brain microsomes added EDTA-Fe(II) complex stimulates lipid peroxidation in the presence of NAD(P)H. The highest level of lipid peroxidation was obtained at a ratio at EDTA:Fe(II) of 1:1 (fig.1.). The NADH-dependent lipid peroxidation was even more intensive than NADPH dependent one and almost reached that of the rather high level observed with liver microsomes (15).

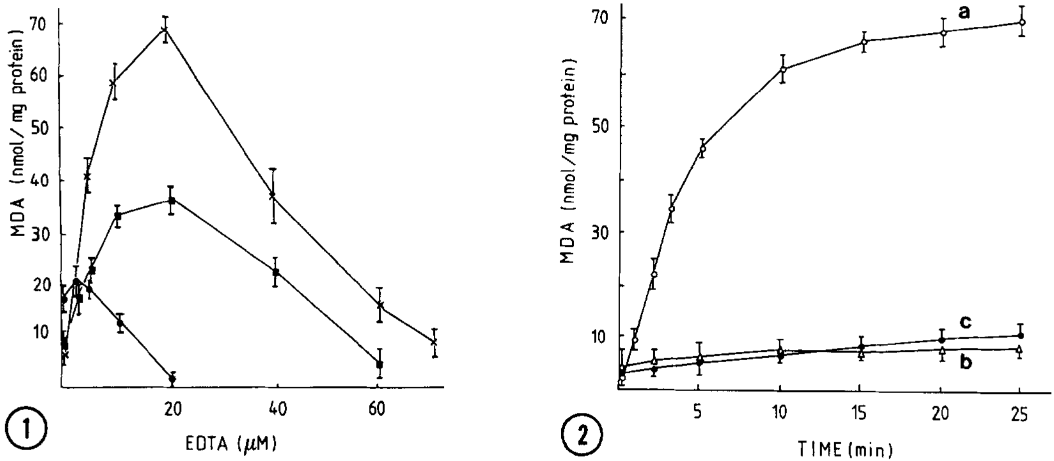

Fig.1. Effect of EDTA/Fe ratio on the lipid peroxidation
The reaction mixture was 50 mM Tris-maleate buffer in the presence of NADH and Fe(III) (●), NADH and Fe(II) (×), NADPH and Fe(II) (■), with various concentration of EDTA.

Fig.2. Requirements for the EDTA-Fe(II) driven lipid peroxidation
a; Standard reaction mixture (see experimental prosedure!) (○), b; standard system -NADH (△), c; standard system with heat-denaturated microsomes (●).

On the other hand, when EDTA-Fe(III) was added at the ratio of 1:1, lipid peroxidation was completely blocked (fig.1.).

Fig.2. illustrates that the EDTA-Fe(II) stimulated lipid peroxidation is a NADH-dependent and enzymatic process (curve a), since either in the absence of NADH (curve b) or in the presence of heat-denaturated microsomes (curve c), the rate of lipid peroxidation was negligible.

The initial rate of EDTA-Fe(II) mediated microsomal lipid peroxidation did not depend on the time of addition of NADH, so the arising initial factor is stabil (Fig.3.).

Table 1. demonstrates that the presence of neither SOD plus catalase nor mannitol had any appreciable effect on the EDTA-Fe(II) enhanced lipid peroxidation, suggesting that superoxide-anion, H_2O_2, or $OH^{\bullet}$ might not play any role in the process.

If a ferrous iron is complexed by a strong field ligand (i.e.EDTA) at neutral pH, it will be oxidized by air due to the decrease of its electrochemical potential (16,7). Under the conditions used the EDTA-Fe(II) complex, contrary to ferrous-ammonium-sulphate suffered therefore, a fast and complete autoxidation and in accordance with this an equivalent oxygen consumption was recorded (not shown).

These observations suggest, that it is the oxidized EDTA-Fe(II) complex which is responsible for the stimulation effect and that this complex is not identical with the complex obtained from EDTA and Fe(III). Since the

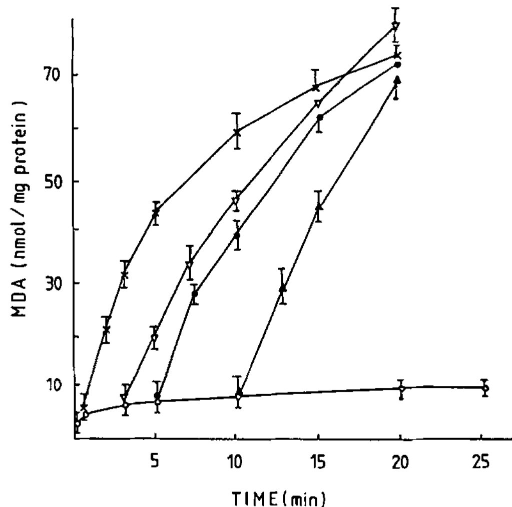

Fig.3. Effect of NADH added in different times
Standard system -NADH (○), +NADH at 0' min. (×), +NADH at 3' min (▽), +NADH at 5' min (●), +NADH at 10' min (▲).

behavior of the central ion and its electrons are strongly affected by its redox state and the ligands, we investigated the Mossbauer and EPR spectra of oxidized EDTA-Fe(II).

Complex chemical aspects

Fig. 4/A shows the typical anisotropic line broadening of EDTA-Fe(III) complex at g_{eff}=4.32.(17,18).
The spectrum of the oxidation product of EDTA-Fe(II) consists of a broad line(peak to peak width about 38 mT) at g_{eff}=4.26 (Fig.4/B.),which did not

Table 1. Inhibition of autoxidated EDTA-Fe(II) stimulated lipid peroxidation

		TBA reactivity	percent of standard
Standard system		68.0+5.5	-
Standard system plus SOD + Catalase(U/ml)			
15	25	63.5+8.0	93
30	50	60.6+6.4	89
60	100	56.1+7.0	83
Mannitol(mM)			
7		69.0+4.0	101
14		68.2+5.1	100
20		70.8+6.2	104

The following additions were made to the incubation mixture as indicated: 0.3 mM NADH, 20 µM EDTA-Fe(II) (1:1), 0.5 mg/ml microsomal protein.
Values are nmole MDA mg protein^{-1}·20 min^{-1}. The data are given the mean of three experiments±S.D.

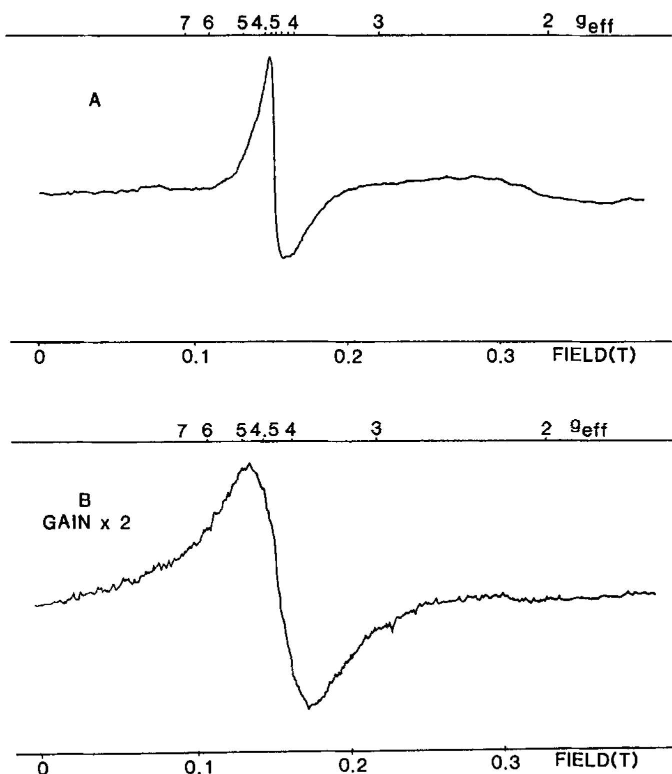

Fig.4. The 80 ^{0}K X-band EPR spectra of EDTA-Fe(III) (A) and oxidized EDTA-Fe(II) (B) in aqueous solution at pH=7. Both concentration was 50 mM.

change in time (within one hour) and was not effected by lowering the concentration of EDTA-Fe(II).

The relaxation time becomes much shorter in the case of oxidized EDTA-Fe(II) resulting in the broadening of the line at g_{eff}=4.3. This broadening can be explained in two ways:either by a dipole interaction between two Fe(III) ions or by the presence of other paramagnetic components (for example O_2 or superoxide anion) in the proximity of Fe(III) ions. The formation of dimers is not likely, because in the oxidized Fe(II) samples no signal at geff=2 could be observed.

The Mossbauer spectrum of frozen EDTA-Fe(III) solution at pH=7 showed a broad single line (Fig.5/A.) when the solution was prepared from $FeCl_3$ and EDTA solution. In this case the line width at the half of the maximum was Γ=2.69 mm s^{-1} and the isomer shift was δ=0.49 mm s^{-1}.

When EDTA-Fe(II) solution was obtained at pH=7 and oxygen bubbling was used for the transformation of EDTA-Fe(II) to EDTA-Fe(III) the Mossbauer spectrum was significantly different (Fig.5/B.). This spectrum was best fitted by a quadrupole splitting of ΔE=0.43 mm s^{-1} with isomer shift of δ=0.54 mm s^{-1} and with line width of Γ=1.43 mm s^{-1}.

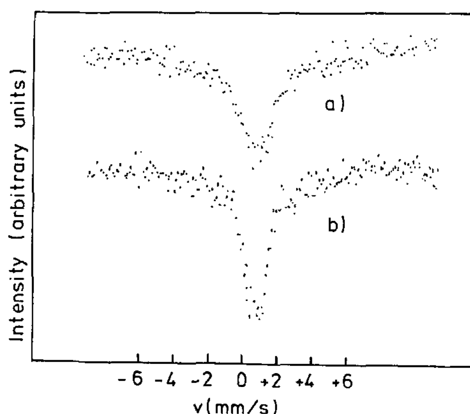

Fig.5. Mossbauer spectra of the frozen solution of o.5 M EDTA-Fe(III)
A. Sample was prepared from $FeCl_3$ and EDTA solution at pH=7.
B. Sample was made from EDTA-Fe(II) by the oxidation with bubbling oxigen at pH=7.
C. Sample was produced by the autoxidation of EDTA-Fe(II) at pH=8.5. The autoxidation took place in 60 minutes.(The doublet of small intensity belongs to the remaining iron(II) compound.)

The characterization of brain microsomal lipid peroxidation induced by EDTA-iron complex showed some significant difference between the effect of EDTA-Fe(III) and EDTA-Fe(II), that could be observed after the oxidation of EDTA-Fe(II) too.The reason of the difference between the two EDTA-iron complexes should be the different chemical structure of the complexes. It is evidenced from the EPR and Mossbauer spectroscopy, that in the EDTA-Fe(III) and the oxidized EDTA-Fe(II) solutions the Fe(III) ion is in different molecular enviroments (19).

During the oxidation of EDTA-Fe(II) the initial agent arising is not in itself able to induce lipid peroxidation. The existence of enzymatic process requiring NADH rather than NADPH was demonstrated, which could explain the redox cycling of oxidized EDTA-iron complex, by the microsomal enzymes.

In the brain microsomes, the EDTA-Fe(III)-oxygen complex, occured in oxidation of EDTA-Fe(II), seems to be a much better substrate for this enzymatic process, than the EDTA-Fe(III). To understand the initiation of lipid peroxidation, further investigations are planed to get more information about the role of microsomal enzyme(s) in the transformation of EDTA-iron complexes.

ACKNOWLEDGEMENT

This study was supported in part by the Association for International Cancer Research.

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