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The mechanism of initiation of lipid peroxidation. Evidence against a requirement for an iron(II)–iron(III) complex

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When Fe^{2+} ions are added to rat-liver microsomes, lipid peroxidation begins after a short lag period. Fe^{2+}-dependent peroxidation in the first few minutes of the incubation can be increased by adding Fe^{3+}, ascorbic acid or Pb^{2+} ions; these stimulations are not additive. By contrast, Pb^{2+} ions inhibit peroxidation of microsomes in the presence of Fe^{3+}/ascorbate or Fe^{3+}-ADP/NADPH. In liposomes made from ox-brain phospholipids, Fe^{2+}-dependent peroxidation is stimulated slightly by Fe^{3+}, but much more so by ascorbic acid, Al^{3+} or Pb^{2+}; these stimulations are not additive. Liposomal peroxidation in the presence of Fe^{3+}/ascorbate is inhibited by Pb^{2+} or Al^{3+}. These results argue against the participation of an Fe^{2+}–Fe^{3+}–O_2 complex, or a critical 1:1 ratio of Fe^{2+} to Fe^{3+}, in the initiation of lipid peroxidation in liposomes and rat-liver microsomes.

INTRODUCTION

Lipid peroxidation is sometimes a major mechanism of cell injury in organisms subjected to oxidative stress (reviewed in [1–3]), although it is by no means the only mechanism of injury [3,4]. However, surprisingly little is known about the chemistry of initiation of peroxidation in membrane systems, such as liposomes or microsomes. Peroxidation in these systems is usually studied after adding iron ions, e.g. Fe^{2+} (sometimes as complexes, e.g. Fe^{2+}–ADP), Fe^{3+} and a reducing agent (e.g. ascorbic acid) or Fe^{3+}–ADP and NADPH (in the case of microsomes). The highly-reactive hydroxyl radical ($OH^{\cdot}$) can often be detected in such systems, and its formation is inhibited by catalase [5–8]. Hydroxyl radical is known to be capable of initiating lipid peroxidation by abstracting hydrogen atoms from fatty-acid side chains [9,10]. However, H_2O_2-degrading enzymes or scavengers of $OH^{\cdot}$ rarely inhibit iron-dependent peroxidation in liposomal or microsomal systems [5–8], suggesting that $OH^{\cdot}$ plays little, if any, role in the observed peroxidation. The $HO_2^{\cdot}$ radical (the protonated form of O_2^{-}) is capable of abstracting hydrogen from polyunsaturated fatty acids [11] or from the lipids present in low-density lipoproteins [12], but superoxide dismutase has little effect on peroxidation in liposomal or microsomal systems, except under conditions in which O_2^{-} is acting to reduce Fe^{3+} to Fe^{2+} [13,14]. Thus $HO_2^{\cdot}$ seems unlikely to play a major role as initiator of peroxidation in these systems.

There have therefore been various suggestions that iron–oxygen species are responsible for initiation. Perferryl radical has been proposed as an initiator (e.g. [15]), but the poor reactivity of perferryl complexes makes this suggestion unlikely [16]. Ferryl species might account for the ability of mixtures of haem proteins and H_2O_2 to stimulate lipid peroxidation (reviewed in [17]), but H_2O_2-degrading enzymes do not usually inhibit iron-dependent peroxidation in microsomes and liposomes (see above). More recently Aust *et al.* [18,19] have proposed that a specific Fe^{2+}–Fe^{3+}–O_2 complex, or at least a 1:1 ratio of Fe^{2+} to Fe^{3+}, acts as initiator of peroxidation in liposomal and microsomal systems. Thus, addition of Fe^{2+} ions to lipids results in a short lag period before peroxidation reaches maximal rates. This lag can be shortened or abolished by addition of Fe^{3+} ions [18,19]. Other investigators have reported similar results in studies of the peroxidation of rat-brain synaptosomes [20], rat liver microsomes [21] and rat kidney brush-border membranes [22].

However, some doubts have been raised about an Fe^{2+}–Fe^{3+}–O_2 complex as a specific initiator of peroxidation. Attempts to isolate such a complex have failed [18,23]. The Fe^{2+}/Fe^{3+} ratios required for maximal stimulation of peroxidation have been reported to vary from 1:1 to 7:1 in different experiments [20], perhaps suggesting that a specific stoichiometric complex is not required. Fe^{3+} ions bound to the chelator nitrilotriacetate were more efficient than 'free' Fe^{3+} ions in stimulating Fe^{2+}-dependent peroxidation of microsomes [21]; it seems unlikely that Fe^{3+} would detach from this strong chelator and form a complex with Fe^{2+}. Borg & Schaich [24] have argued that Fe^{3+} is acting by displacing Fe^{2+} from binding sites on the membrane at which it is acting as a 'radical trap' for peroxyl and alkoxyl radicals.

We have observed that Pb^{2+} ions accelerate Fe^{2+}-stimulated peroxidation in liposomes, and in microsomes at pH 7.4 [26]. Al^{3+} ions are also often stimulatory [25, 26]. In the present paper, we show that, under certain conditions, Al^{3+} and Pb^{2+} ions are able to replace Fe^{3+} in accelerating peroxidation during the lag period in Fe^{2+}-stimulated peroxidation in liposomes or microsomes, indicating that a specific Fe^{2+}–Fe^{3+} complex cannot be

Abbreviation used: TBA, thiobarbituric acid.

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required for this purpose. Studies with ascorbic acid-stimulated peroxidation are also reported and are consistent with this conclusion.

MATERIALS AND METHODS

Ox-brain phospholipids were prepared as described in [27] and used to form liposomes as described in [25]. Rat liver microsomes were prepared by standard techniques of differential pelleting [26]; the final microsomal pellet was resuspended in 0.25 M-NaCl. All other reagents were of the highest quality available from BDH Chemicals Ltd. or from Sigma. Fe^{2+} was used as ferrous sulphate, Fe^{3+} as ferric chloride, Pb^{2+} as lead acetate or $Pb(NO_3)_2$ and Al^{3+} as aluminium sulphate.

Peroxidation of liposomes

Reaction mixtures contained, in a final volume of 1.0 ml, 0.5 ml of phosphate/saline buffer (3.4 mM-Na_2HPO_4/NaH_2PO_4/0.15 M-NaCl, pH 7.4), 0.2 ml of liposomes (5 mg/ml), and metal ions as stated. Peroxidation was started by adding iron salts (freshly made up in N_2-sparged water), and tubes were incubated at 37 °C for the time stated. Lipid peroxidation was measured as thiobarbituric acid (TBA) reactivity. TBA [0.5 ml; 1 % (w/v)] in 0.05 M-NaOH and 0.1 ml of a 2 % (w/v) solution of butylated hydroxytoluene in ethanol were added, followed by 0.5 ml of 25 % (v/v) HCl. Tubes were heated at 100 °C for 10 min and the chromogen was extracted into 3 ml of butan-1-ol. The absorbance of the upper (organic) layer was read at 532 nm.

Peroxidation of microsomes

Peroxidation was studied in reaction mixtures containing, in a final volume of 1.0 ml, phosphate/saline buffer (described above), pH 7.4, 0.1–0.5 mg (usually 0.25 mg) of microsomal protein, and metal ions as stated. Tubes were incubated at 37 °C for the time stated and analysed for TBA reactivity as described above.

Measurement of Fe^{2+} oxidation

Fe^{2+} oxidation was measured by the ferroxidase assay, in which the absorbance change upon the binding of Fe^{3+} to transferrin is followed [28].

RESULTS

Microsome experiments

Peroxidation was measured by the TBA test. This test measures not only peroxidation occurring in the experiment itself, but also peroxidation taking place during the acid-heating stage of the assay [29,30]. In order to avoid any confusion, the TBA test was performed in the presence of the antioxidant butylated hydroxytoluene, to inhibit peroxidation during the assay itself [30].

When Fe^{2+} or Fe^{2+}-ADP are added to freshly-prepared rat liver microsomes at pH 7.4, the rate of formation of TBA-reactive substances begins slowly, but then accelerates. The length of this lag period varied from preparation to preparation, but was usually between 1 and 7 min (mean 4.2 min in 15 experiments). Fig. 1 shows results from an experiment in which the lag period was 7 min, and Table 1 depicts results from an experiment in which it was 3 min (the variability between experiments is described in the legend to Fig. 1).

In agreement with previous reports [18–22], addition of Fe^{3+} stimulated peroxidation during the lag period, increasing the extent of peroxidation in the first few minutes of the incubation. As expected [26], Pb^{2+} stimulated the Fe^{2+}-dependent peroxidation (Table 1; Fig. 1). It stimulated peroxidation during the lag period as well as or better than Fe^{3+} (Table 1; Fig. 1). The stimulation was not additive to that seen with Fe^{3+}: $Fe^{2+}/Fe^{3+}/Pb^{2+}$ mixtures showed about the same extent of peroxidation as Fe^{2+}/Pb^{2+} mixtures (Table 1; Fig. 1). This was not because peroxidation had reached a maximum; maximum peroxidation in the assay mixtures used here gave $A_{532} > 1.5$. Fe^{3+} or Pb^{2+} alone gave no significant peroxidation in freshly-prepared rat liver microsomes, nor did mixtures of Pb^{2+} and Fe^{3+}.

The lag period could be virtually abolished by starting peroxidation with an Fe^{3+}/ascorbic acid mixture (both 100 μM) instead of Fe^{2+}. In these experiments, Pb^{2+} had little stimulatory effect. Indeed, it inhibited peroxidation (Fig. 1; Table 1). Similarly, Pb^{2+} inhibited the peroxidation observed in the presence of 100 μM-Fe^{3+}–ADP

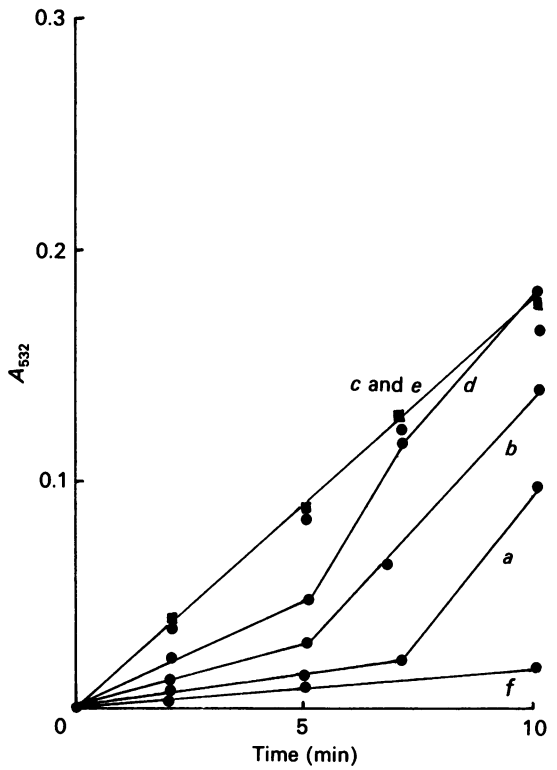

Fig. 1. Time course of Fe^{2+}-stimulated peroxidation in rat liver microsomes at pH 7.4

The reaction mixture contained 0.25 mg of microsomal protein. Peroxidation was measured by the TBA test. In 15 similar experiments, peroxidation at the end of the lag period (as A_{532}) was 0.003–0.106; the lag period varied from 1–7 min. The mean percentage stimulation (at the end of the lag period) by Fe^{3+} was 242 %, that by Pb^{2+} was 436 % and that by both was 449 % (not significantly different from stimulation by Pb^{2+} alone). Line *a*, 100 μM-Fe^{2+} present; line *b*, 100 μM-Fe^{2+} + 100 μM-Fe^{3+}; line *c*, (●), 100 μM-Fe^{2+} + 400 μM-Pb^{2+}; line *d*, 100 μM-Fe^{2+} + 400 μM-Pb^{2+} + 100 μM-Fe^{3+}; line *e*, (■), 100 μM-Fe^{3+} + 100 μM-ascorbate; line *f*, 100 μM-Fe^{3+} + 100 μM-ascorbate + 400 μM-Pb^{2+}. Control experiments with Fe^{3+}, or Pb^{2+} in the absence of Fe^{2+} gave no significant peroxidation (Table 1 shows an example).

Table 1. Peroxidation of rat-liver microsomes at pH 7.4

The reaction mixture contained 0.25 mg of microsomal protein. Peroxidation was started by adding Fe^{2+} (final concentration of 100 μM) or Fe^{3+} and ascorbate (both 100 μM). Tubes were incubated at 37 °C for various times. Samples were taken every 2–3 min. Where indicated, 100 μM-Fe^{3+} or 400 μM-Pb^{2+} were also added. Absorbances are the means of duplicate determinations that differed by no more than 8 %. The lag period in this experiment lasted 3.0 min, so that values after 3 min incubation are presented. The legend to Fig. 1 summarizes results from 15 different experiments.

Metal added to reaction mixture	Extent of peroxidation (A_{532})
None	0.000
Fe^{2+}	0.006
Fe^{3+}	0.001
Pb^{2+}	0.001
Fe^{3+}/Pb^{2+}	0.001
Fe^{3+}/ascorbate	0.071
Fe^{2+}/Fe^{3+}	0.019
Fe^{2+}/Pb^{2+}	0.066
$Fe^{2+}/Fe^{3+}/Pb^{2+}$	0.064
Fe^{3+}/ascorbate/Pb^{2+}	0.008

plus 300 μM-NADPH (results not shown). Although Al^{3+} is well known to stimulate Fe^{2+}-dependent peroxidation [25,26], it also inhibited microsomal peroxidation in the presence of Fe^{3+}/ascorbate or Fe^{3+}-ADP/NADPH (results not shown).

Control experiments proved that Pb^{2+} or Al^{3+} ions had no effect on the TBA test itself, nor did they alter the rate of oxidation of Fe^{2+} ions at pH 7.4 as followed by the binding of the resulting Fe^{3+} to transferrin [28].

Liposome experiments

Previous studies on the role of Fe^{2+}–Fe^{3+}–O_2 complexes on liposomal lipid peroxidation have used liposomes made from extracted microsomal lipids [19]. In the present study, liposomes made from ox-brain phospholipids were used, to provide a different lipid substrate. Table 2 shows a typical experimental result (one out of 32 experiments) in which peroxidation was studied over a 10 min incubation period, during the lag period for Fe^{2+}-stimulated peroxidation (see legend to Table 2). Fe^{3+} produced no peroxidation itself, but it slightly stimulated Fe^{2+}-dependent peroxidation, to an extent which increased with Fe^{3+} concentration. Both Al^{3+} and Pb^{2+} also stimulated peroxidation. Pb^{2+} was more stimulatory than Al^{3+}, in agreement with previous work [25, 26]. In the presence of Al^{3+} or Pb^{2+}, Fe^{3+} gave little or no further stimulation of peroxidation. A striking stimulation of peroxidation, greater than that produced by any concentration of Fe^{3+} tested, was observed using Fe^{3+}/ascorbate. Neither Pb^{2+} nor Al^{3+} produced any further stimulation in reaction mixtures containing Fe^{3+}/ascorbate; they inhibited the peroxidation reproducibly.

Table 2. Action of Fe^{3+}, Al^{3+} and Pb^{2+} on peroxidation of ox-brain phospholipid liposomes

Peroxidation in the presence of liposomes, buffer at pH 7.4 and, where indicated, Fe^{2+} (100 μM), Fe^{3+} (concentration indicated), ascorbic acid (100 μM), Pb^{2+} (400 μM) or Al^{3+} (400 μM), was measured at 1–2 min intervals during incubation at 37 °C by the TBA test in the presence of butylated hydroxytoluene. In this experiment, the lag period lasted 10 min, and values after 10 min incubation are presented. In a series of 32 experiments, peroxidation at the end of the lag period varied from 0.086 to 0.152. Mean percentage stimulations by 100 μM-Fe^{3+}, 400 μM-Pb^{2+} and 400 μM-Al^{3+} were 67 %, 130 % and 89 % respectively.

Metal added to reaction mixture	Extent of peroxidation (A_{532})
None	0.000
Fe^{2+}	0.095
Fe^{3+} (100–400 μM)	0.000
Fe^{2+}/Fe^{3+} (100 μM)	0.126
Fe^{2+}/Fe^{3+} (10 μM)	0.102
Fe^{2+}/Fe^{3+} (50 μM)	0.114
Fe^{2+}/Fe^{3+} (80 μM)	0.121
Fe^{2+}/Fe^{3+} (200 μM)	0.127
Fe^{2+}/Fe^{3+} (300 μM)	0.179
Pb^{2+}	0.000
Fe^{2+}/Pb^{2+}	0.266
$Fe^{2+}/Pb^{2+}/Fe^{3+}$	0.183
Fe^{2+}/Al^{3+}	0.213
Al^{3+} (100 μM)	0.000
$Fe^{2+}/Al^{3+}/Fe^{3+}$ (100 μM)	0.234
Fe^{3+}/ascorbate	0.480
Fe^{3+}/ascorbate/Pb^{2+}	0.170
Fe^{3+}/ascorbate/Al^{3+}	0.117

DISCUSSION

It has been proposed that a critical 1:1 ratio of Fe^{2+} to Fe^{3+} is needed to stimulate lipid peroxidation maximally, perhaps by formation of an Fe^{2+}–Fe^{3+}–O_2 complex [18, 19,31]. Thus when peroxidation is started by adding Fe^{2+}, the lag period is thought to be due to the time taken for some Fe^{3+} to form by oxidation of Fe^{2+}. Ascorbate would stimulate peroxidation by reducing some Fe^{3+} to Fe^{2+} to give the optimal 1:1 Fe^{2+}/Fe^{3+} ratio for initiation [18,19,23,31]. NADPH added to microsomes would provide electrons for reduction of some Fe^{3+}–ADP in a similar way. However, in our experiments with liposomes and microsomes (Tables 1 and 2, Fig. 1), ascorbate-stimulated peroxidation proceeded much faster than peroxidation stimulated by Fe^{2+}/Fe^{3+}, suggesting that ascorbate cannot be acting only by giving an equimolar Fe^{2+}/Fe^{3+} ratio for the inhibition process.

In rat-liver microsomes, Fe^{2+}-dependent peroxidation is stimulated by Pb^{2+} and by Fe^{3+}: these stimulations are not additive. Pb^{2+} did not interfere with the assay systems, nor did it cause accelerated oxidation of Fe^{2+} to Fe^{3+}. It is notable that Pb^{2+} inhibited peroxidation when Fe^{3+}/ascorbate was present; similar results have been found for Al^{3+}, which also stimulates Fe^{2+}-dependent peroxidation. It follows that Al^{3+} and Pb^{2+} can replace Fe^{3+} as stimulators of Fe^{2+}-dependent peroxidation. Thus, whatever Fe^{3+} is doing in this system, it cannot be acting by forming a specific complex that is required for peroxidation. Our data are more consistent with the suggestion of Borg & Schaich [24] that Fe^{3+} interferes with inhibitory reactions of Fe^{2+} by displacing it from certain sites on the membrane: Pb^{2+} and Al^{3+} might also

be able to do this. Why they should inhibit Fe^{3+}/ascorbate-dependent peroxidation is unclear, but this observation illustrates the complexity of these systems.

With ox-brain phospholipid liposomes, Fe^{3+} had a smaller stimulatory effect on Fe^{2+}-dependent peroxidation than in microsomes. Pb^{2+} and Al^{3+} had greater stimulatory effects, as did ascorbic acid. Again, stimulations by Pb^{2+} or Al^{3+} and Fe^{3+} were not additive, suggesting that Pb^{2+} and Al^{3+} are doing whatever Fe^{3+} is doing (although rather more effectively in this system).

We conclude that, since the action of Fe^{3+} can be replaced by other metal ions, a critical 1:1 Fe^{2+}/Fe^{3+} ratio, or a specific Fe^{2+}–Fe^{3+}–O_2 complex, cannot be required for the initiation of lipid peroxidation. Indeed, it has been pointed out that, since microsomes and liposomes always contain traces of pre-formed lipid peroxides, added iron ions are more likely to stimulate peroxidation by decomposing these to chain-propagating peroxyl and alkoxyl radicals rather than by generating initiating species [24,32,33]. A study of initiation requires the use of peroxide-free lipid systems, which are difficult to obtain in biochemical systems [32].

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