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Microsomal Lipid Peroxidation

I. Characterization of the Role of Iron and NADPH

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SUMMARY

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The NADPH/iron-dependent peroxidation of lipids in rat liver microsomes was found to depend on the presence of free ferrous iron. High concentrations of Fe^{2+} initiated microsomal lipid peroxidation at an initial rate which was independent of NADPH. With Fe^{3+}, lower Fe^{2+} concentrations, or longer incubation times, NADPH stimulated microsomal lipid peroxidation and this stimulation was blocked by cytochrome *c*, which is capable of accepting electrons from the microsomal NADPH–cytochrome *P*-450 reductase. NADPH did not induce peroxidation in the absence of iron, nor did ferric iron in the absence of NADPH. Thus the role of NADPH appears to be maintenance of iron in the reduced Fe^{2+} state. The presence of superoxide dismutase, catalase, or the hydroxyl radical scavengers ethanol or thiourea had little or no effect on the iron-initiated peroxidation, ruling out dependence on reduced states of oxygen. Complete chelation of iron by EDTA completely inhibited peroxidation and no stimulation by either EDTA or ADP was observed over the peroxidation produced by free iron alone. Preincubation of microsomes in the absence of iron did not enhance the rate of peroxidation upon subsequent addition of iron, suggesting that iron acts by initiating peroxidative decomposition of membrane lipids rather than by catalyzing the breakdown of preformed hydroperoxides. Liposomes of extracted microsomal lipid also underwent peroxidation in the presence of ferrous iron at a rate comparable to that of intact microsomes. Ascorbate stimulated iron-induced liposomal peroxidation but had no effect in the absence of iron. Hydrogen peroxide did not enhance iron-induced liposomal peroxidation and inhibited at high concentrations, ruling out a role for hydroxyl radicals produced by reduction of H_2O_2 by Fe^{2+}.

INTRODUCTION

A number of pathological conditions stemming primarily from exposure to toxic chemicals are believed to be manifested through a process of lipid peroxidation (1). This phenomenon has been most extensively studied in the liver, where peroxidative decomposition of cellular lipids has been associated with functional disturbances in subcellular structures.

The process of lipid peroxidation in isolated liver microsomes has been shown to require NADPH and molecular oxygen and to be markedly stimulated by iron salts (2–5). The involvement of NADPH in the peroxidative process is mediated by the microsomal NADPH–cytochrome *P*-450 reductase, the purified form of which has also been shown to promote NADPH- and iron-dependent peroxidation of liposomes (6). The nature of the interaction between iron and NADPH which leads to peroxidation of lipids is unclear, however, Early evidence had suggested that nonheme iron would be active if present as a phosphate complex (2), although the requirement for phosphate was later disproved (5). Other observations of the effectiveness of inorganic iron led to speculation that its involvement might be simply to stimulate decomposition of lipid hydroperoxides (7), since it is well known that many transition metals accelerate homolysis of peroxidic materials (8). Due to the lack of direct evidence, however, it remains uncertain whether this process plays an important role in the peroxidation of microsomal lipids.

Current views on the action of iron have focused on its

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possible role in initiating the lipoperoxidative sequence. Some authors have postulated a direct electron transfer from NADPH to Fe^{3+}, catalyzed by the reductase,[1] which yields Fe^{2+} as the initiator and propagator of microsomal peroxidation (9, 10). Other authors, however, have proposed a more complicated involvement of the reductase in the production of superoxide anions ($O_2^-\cdot$), which dismutate to hydrogen peroxide (H_2O_2) and react with Fe^{2+} to yield hydroxyl radicals ($OH\cdot$) as the ultimate initiator of peroxidation (11, 12).

We have attempted to distinguish between the various possible mechanisms of iron- and NADPH-stimulated lipid peroxidation in the experiments reported here. The evidence which we present strongly favors Fe^{2+} as the active initiating species and shows that reductase-generated superoxide is not essential since high concentrations of Fe^{2+} promote microsomal lipid peroxidation at the same initial rate in the presence or absence of NADPH. In addition, it appears that only free Fe^{2+} is stimulatory, whereas EDTA-chelated iron is not effective.

EXPERIMENTAL PROCEDURES

Materials. NADPH, ADP, ascorbic acid, EDTA, $FeSO_4$, $Fe(NO_3)_3$, epinephrine, trichloroacetic acid, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, bovine serum albumin, xanthine oxidase (grade I; buttermilk), cytochrome *c* (type VI; horse heart), and superoxide dismutase (type I; bovine blood) were obtained from Sigma Chemical Company, St. Louis, Missouri. Tetraethoxypropane was purchased from Tridom Chemical, Hauppauge, New York; thiourea and 2-thiobarbituric acid were obtained from Eastman Chemicals, Rochester, New York. Fatty acid methyl ester standards were products of Nu Chek Prep, Elysian, Minnesota.

Preparation of microsomes. Adult male Long–Evans rats, 200–300 g, were sacrificed by intraperitoneal injection of 40 mg of sodium pentobarbital.[2] Livers were removed and minced thoroughly with scissors. The minced tissue was washed several times with ice-cold buffered potassium chloride (0.15 M KCl, 5 mM Tris–maleate, pH 7.42) containing 1 mM EDTA and homogenized in the same, using a Teflon–glass homogenizer. The homogenate was centrifuged successively at 300, 1600, 8000, and 30,000*g* for 10–15 min at each speed, and each of the pellets was discarded. Microsomes were obtained from the 30,000*g* supernatant by centrifugation at 100,000*g* for 1 h. The pellet was washed once by suspension in and resedimentation from buffered potassium chloride with no EDTA, resuspended in the same at a concentration of 2–3 mg protein/ml, and stored in liquid nitrogen. This washing procedure was performed with volumes which were adequate to assure that less than 10^{-2} μmol EDTA was present in the microsomal incubations.

Protein concentrations were determined by the Lowry method (13). Purity and integrity of the microsomes were checked by routinely assaying for NADPH–cytochrome *c* reductase activity, which was measured spectrophotometrically by recording the optical density change at 550 nm due to reduction of cytochrome *c* (14). Activity of the liver microsomal enzyme was 213 ± 34 nmol/min/mg protein.

Preparation of liposomes. Lipids were extracted from microsomes by the method of Bligh and Dyer (15) and the chloroform phase was completely evaporated under nitrogen gas. The resulting lipid film was hydrated for 20 min followed by ultrasonic dispersion under N_2 for 1 min or until a clear uniform suspension was obtained.

Incubation procedure. Microsomes or liposomes were incubated for the specified lengths of time at 37°C. The total volume was usually 1.0 ml (or occasionally 0.5 ml) and contained 40 mM Tris–maleate buffer (pH 7.4) unless otherwise noted. Iron was added either as $Fe(NO_3)_3$ or as $FeSO_4$. Due to the instability of iron in solution, $Fe(NO_3)_3$ was prepared fresh and used immediately; $FeSO_4$ stock solutions were stabilized by acidifying with 0.1 mol of HCl/mol of iron, although when higher concentrations were used, a nonacid solution was freshly prepared so as not to perturb the pH of the buffered incubation mixture.

The xanthine oxidase system used to induce lipid peroxidation consisted of 0.8 mg (0.53 unit) of xanthine oxidase and 5 mM acetaldehyde. Superoxide production by this system as well as by liver microsomes was measured by monitoring adrenochrome formation from epinephrine (4 mM) at 480 nm as described by Aust *et al.* (16). An extinction coefficient of 4.02×10^3 M^{-1} cm^{-1} was used.

Additional reagents were included as described in the figure legends.

Measurement of lipid peroxidation. Lipid peroxidation was quantitated by colorimetric determination of malondialdehyde formation after complexing with thiobarbituric acid (17). Peroxidation was stopped by the rapid addition of 50 μl of 0.2% butylated hydroxytoluene in ethanol followed by 300 μl of 20% trichloroacetic acid and 600 μl of 0.05 M TBA (or half these volumes when the incubation volume was 0.5 ml). Bovine serum albumin (0.5 mg) was added to facilitate precipitation of protein during a 10-min centrifugation, following which the resulting clear supernatant was removed and delivered to clean screw-cap culture tubes which were then tightly capped and boiled for 8 min. The amount of colored product was measured by determining absorbance spectrophotometrically at 532 nm and converting to nanomoles of MDA using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}. This value was verified using a tetraethoxypropane standard. None of the reagents present in the incubations affected the tetraethoxypropane-derived color. The absorbance spectrum for color produced during microsomal (or liposomal) incubations showed a maximum at 532 nm and thus represented true MDA–TBA color.

Fatty acid analysis. In some experiments, fatty acids

[1] Abbreviations used: reductase, NADPH–cytochrome *P*-450 (or cytochrome *c*) reductase; MDA, malondialdehyde; TBA, thiobarbituric acid; SOD, superoxide dismutase.

[2] Comparison of liver microsomes from pentobarbital-treated rats with microsomes from rats killed by cervical dislocation showed no difference in the rate of lipid peroxidation at several different iron concentrations and also no effect on NADPH–cytochrome *c* reductase activity. Pentobarbital sacrifice was used because of its greater convenience and reproducibility.

were quantified following incubation of microsomes under the specified lipid peroxidizing conditions. Aliquots of the incubation mixture were removed for MDA determinations just prior to stopping peroxidation by the addition of 6 ml of 2:1 methanol–chloroform. Lipid was extracted by the Bligh and Dyer method (15), and fatty acid methyl esters were prepared as described by Mavis and Vagelos (18). Relative weight percentages of the fatty acids present in the final extract were determined by gas–liquid chromatography (Hewlett Packard, Model 5830A) and identified by comparison to a series of methyl ester standards.

RESULTS

Incubation pH and buffer optima. Considerable differences in iron-dependent lipid peroxidation were observed with different buffer systems. When Tris–maleate was used, high rates of lipid peroxidation were produced by relatively low iron concentrations, as shown in Fig. 1. A similar dependence on iron was observed with Tris–HCl (data not shown). In contrast, phosphate buffer greatly suppressed iron-induced peroxidation such that it was necessary to lower the phosphate concentration to less than 8 mM in order to observe stimulation of peroxidation by 1 μM $FeSO_4$ (data not shown). At higher phosphate concentrations, greater amounts of iron were required to stimulate peroxidation. This effect may be due to the formation of a weak association between iron and phosphate which may impair the reactivity of iron toward microsomal lipids.

Optimal rates of MDA formation were obtained with 40–80 mM Tris–maleate, pH 7.4, although varying the pH between 6.4 and 8.0 had little effect on the rate of lipoperoxidative activity. At lower concentrations of (or in the absence of) Tris–maleate, less peroxidation was observed. However, in the presence of an amount of KCl (0.4 M) sufficient to maintain the ionic strength (and a small amount of phosphate buffer to stabilize the pH), the addition of Tris buffer up to 80 mM had no effect on peroxidation. Thus, stimulation of peroxidation by higher Tris concentrations in the absence of KCl can be attributed to an ionic strength effect. It has been previously demonstrated that the lipid peroxidation reaction catalyzed by the purified reductase requires a high ionic strength (6).

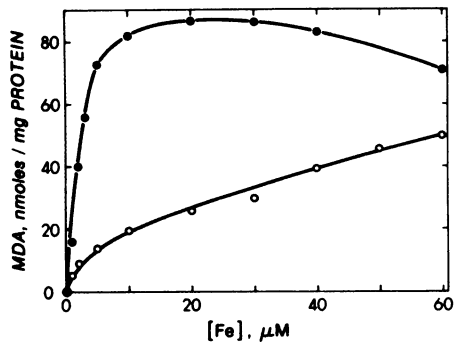

FIG. 1. *Effect of iron concentration on MDA formation in liver microsomes*

Microsomal protein ranging from 45 to 160 μg was incubated with 200 uM NADPH and either $FeSO_4$ (●) or $Fe(NO_3)_3$ (○) for 15 min. Other conditions were as described in Experimental Procedures. Each point represents the mean value from two to four experiments.

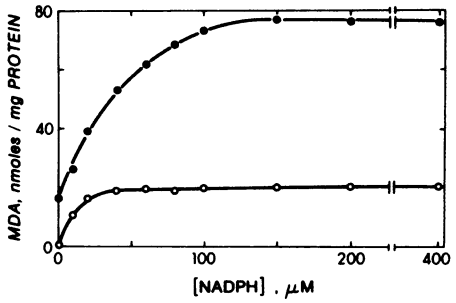

FIG. 2. *Effect of NADPH concentration on lipid peroxidation in liver microsomes as stimulated by 10 μM $FeSO_4$ (●) or 1 μM $FeSO_4$ (○)*

Microsomes (99–133) μg of protein) were incubated for 15 min with the indicated amounts of NADPH. Each point represents the mean value from two experiments.

Characterization of iron and NADPH dependences. Microsomes prepared in the absence of EDTA peroxidized at a rapid rate upon the addition of NADPH. This activity was completely inhibited by EDTA, indicating a dependence on free metal ions. Consequently, microsomes were prepared in the presence of EDTA followed by washing to remove EDTA in order to eliminate metal ions and thus allow control over their concentration. Figure 1 shows that peroxidation of these microsomes in the presence of NADPH was absolutely dependent upon added iron. Concentrations of ferrous iron as low as 0.5 μM were highly stimulatory and a maximal rate was obtained at 20 μM. Similar concentrations of ferric iron were nearly fourfold less effective.

Figure 2 illustrates the dependence of microsomal lipid peroxidation on NADPH at two different concentrations of ferrous iron. With 10 μM $FeSO_4$, increasing NADPH concentration enhanced the rate of lipid peroxidation up to 150 μM NADPH, where the amount of MDA produced was about that expected for complete reaction of membranous polyunsaturated fatty acids (see Table 2). At 1 μM $FeSO_4$, a much lower concentration of NADPH (40 μM) was apparently saturating, and the magnitude of MDA formation associated with NADPH saturation was four times less than with 10 μM ferrous iron. Even a 10-fold increase in NADPH above 40 μM at the lower iron concentration did not produce any increase in the rate of peroxidation, which suggests that the Fe^{2+} concentration is the primary determinant of the rate of peroxidation. In fact, with 10 μM $FeSO_4$, a significant amount of peroxidation occurred in the absence of NADPH.

Characterization of the time course of this NADPH-independent microsomal lipid peroxidation stimulated by 20 μM ferrous iron revealed a rapid initial rate of peroxidation which subsided after about 5–10 min (data not shown). In contrast, the same concentration of ferric iron resulted in over a 10-fold lower initial rate, and the extent of peroxidation after 60 min was only about 25% of that produced by $FeSO_4$. The ability of Fe^{2+} to promote a high initial rate of peroxidation in the absence of NADPH suggests that ferrous iron is capable of directly initiating the peroxidative process. However, this effect is appar-

ently not catalytic since the reaction subsides prior to extensive depletion of membrane lipid substrate. Therefore, enhancement of peroxidation by NADPH (Fig. 2) may be simply due to its ability to stimulate reduction of Fe^{3+} to Fe^{2+}, as catalyzed by the microsomal reductase, thereby regenerating the active reduced state of iron.

In support of this mechanism, it was found that the rate of NADPH-dependent lipid peroxidation initiated by 1 μM $FeSO_4$ was over 75% inhibited by 200 μM cytochrome *c*, added either at zero time or after 15 min of peroxidation. In a related series of experiments, cytochrome *c* was observed to partially inhibit Fe^{2+}-stimulated peroxidation in the absence of NADPH, an effect which was attributed to its ability to oxidize ferrous iron directly to the inactive ferric state. However, NADPH-dependent peroxidation was inhibited to a much greater extent by cytochrome *c*, and this additional inhibition probably represents a competition between reduction of cytochrome *c* and reduction of iron by the microsomal reductase.

Conversely, it was found that 200 μM Fe^{3+} produced a 25% inhibition of microsome-catalyzed reduction of cytochrome *c* by NADPH, presumably by competing with cytochrome *c* as an acceptor of electrons from the reductase. This effect was not attributable to direct oxidation of cytochrome *c* by Fe^{3+} since Fe^{3+} did not diminish the spontaneous reduction of cytochrome *c* in the absence of microsomes. The addition of 1.0 mM EDTA had no effect on reductase activity in the absence of iron, but appeared to enhance the inhibitory properties of 200 μM $Fe(NO_3)_3$, possibly by stabilizing the ferric iron in a form which is more readily reduced.

We believe that the data in Fig. 3 clearly show that NADPH is required only for maintenance of iron in the ferrous state. Using short incubation times and increasing concentrations of iron in the presence and absence of NADPH, it was found that at 40–50 μM Fe^{2+}, the initial rate of lipid peroxidation is independent of NADPH. Thus ferrous iron alone is capable of promoting lipid peroxidation at a rate comparable to that occurring in the presence of NADPH if conditions are chosen such that regeneration of Fe^{2+} from Fe^{3+} is not required. The lack of stimulation by NADPH under these conditions seems to contradict previous suggestions that reductase-generated superoxide anions lead to the formation of hydroxyl radicals (11, 12) which directly initiate lipid peroxidation. Since sufficient amounts of reduced iron obviate the requirement for any input from the reductase, it appears that if activated oxygen is in fact produced through NADPH oxidation, it does not initiate lipid peroxidation beyond that induced by iron alone.

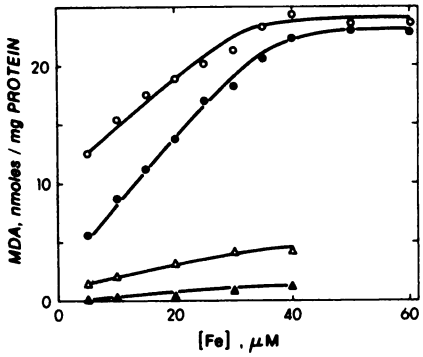

FIG. 3. *Effect of increasing concentrations of Fe^{2+} and Fe^{3+} with and without NADPH on initial rates of lipid peroxidation in rat liver microsomes*

Microsomes (95–160 μg of protein) were incubated with either $FeSO_4$ with no NADPH (●), $FeSO_4$ with 200 μM NADPH (○), $Fe(NO_3)_3$ with no NADPH (▲), or $Fe(NO_3)_3$ with 200 μM NADPH (△). Time of incubation was 100 s. Other conditions are described in Experimental Procedures. Each point represents the mean value from two to five experiments. Points at 50 and 60 μM $FeSO_4$ are not statistically different at $P = 0.80$, whereas points ≤40 μM $FeSO_4$ are statistically different at $P = 0.20$ or less (as calculated using a two-tailed *t* test).

Further evidence for the lack of involvement of activated oxygen in the initiation of microsomal lipid peroxidation was obtained by incubating microsomes in the presence of activated oxygen scavengers. According to the mechanism suggested by Lai and Piette (12), reductase-mediated oxidation of NADPH gives rise to $O_2^-\cdot$ which spontaneously dismutates to H_2O_2. The hydrogen peroxide thus generated undergoes a Fenton reaction with reduced metal ions (i.e., Fe^{2+}) to yield OH· as the ultimate initiator of lipid peroxidation. Contrary to this hypothesis, we found that the addition of superoxide dismutase (50–90 units), which would be expected to promote peroxidation by increasing the rate of $O_2^-\cdot$ dismutation to H_2O_2, had no effect on microsomal peroxidation even though the iron concentration was low and the system was highly NADPH dependent (data not shown). Thus neither the initiation of peroxidation nor the reduction of iron by NADPH appears to involve the superoxide anion. The highest amount of SOD tested was capable of completely inhibiting adrenochrome formation from epinephrine as stimulated by 0.53 unit of xanthine oxidase (data not shown; see Experimental Procedures) and was thus highly active. These results confirm previous reports on the lack of effect of SOD on lipid peroxidation (9). Similarly, the addition of catalase (550 units), alone or in combination with SOD, had no effect on NADPH/Fe^{2+}-stimulated MDA formation, which is inconsistent with a H_2O_2 intermediate, as postulated by Lai and Piette (12). Moreover, amounts of ethanol (1–2%) and thiourea (4–8 mM) which have been reported to effectively scavenge hydroxyl radicals (19) did not inhibit NADPH-dependent peroxidation, although thiourea was observed to cause a slight reduction in NADPH-independent peroxidation and may, therefore, have weak antioxidant properties.

Mitochondria contain an active superoxide dismutase (20) and are variably present in different microsomal preparations. Thus it was considered that the lack of effect of activated oxygen scavengers on microsomal lipid peroxidation might be due to mitochondrial SOD contamination acting to obscure the effect of SOD or hydroxyl radical scavengers added to our microsomes. However, no activity of the mitochondrial marker, succinate–cytochrome *c* reductase, was detectable in our microsomes. Also, using the inhibition of xanthine oxidase-induced conversion of epinephrine to adrenochrome to quantitate the presence of SOD, we determined that the

SOD content of our microsomes was less than 10^{-2} units/μg of protein. This amount of SOD is apparently insufficient to block superoxide-dependent phenomena since nearly 50 units of added SOD was required to inhibit xanthine oxidase-induced microsomal lipid peroxidation completely (see Table 1). Furthermore, in the presence of NADPH, these same microsomes stimulated epinephrine oxidation at a higher rate than that reported by other investigators (16), and this response was over 50% inhibited by 7.5 units of added SOD, thus demonstrating the viability of superoxide in our microsomal incubations in the absence of added SOD.

The possibility that Tris, with its ethanolic residues, was acting as a hydroxyl radical scavenger and thereby preventing hydroxyl radical-mediated peroxidation was also tested. Microsomes were prepared in the absence of Tris buffer and incubated with 1 μM $FeSO_4$, 300 μM NADPH, 3 mM potassium phosphate (pH 7.4), 0.4 M KCl, and various concentrations of Tris–maleate (pH 7.4). Under these conditions, Tris had no effect on lipid peroxidation over the concentration range of 0.2 to 80 mM. Moreover, the addition of ethanol (1%) or thiourea (8 mM) to Tris-free microsomes did not inhibit peroxidation. These results provide further support for the lack of involvement of OH· in NADPH-dependent microsomal peroxidation and justify the use of Tris buffers in the present studies as well as in those of other investigators (6, 9–11).

Table 1 shows the ability of xanthine oxidase-generated O_2^-· to induce microsomal lipid peroxidation. In the absence of iron, the addition of the xanthine oxidase system was associated with a relatively low level of lipid peroxidation which was not inhibited by SOD. Likewise, iron alone (1 μM) did not induce extensive peroxidation, but when xanthine oxidase and iron were both present, a greater than additive amount of peroxidation was obtained. These results appear to support the contention that superoxide radicals are converted to peroxidation-initiating species of activated oxygen in the presence of reduced iron. However, the addition of SOD, which theoretically should have increased the conversion of O_2^-· to the postulated H_2O_2 intermediate (11, 12), had an inhibitory effect on the xanthine oxidase/Fe^{2+}-induced peroxidation. In addition, thiourea did not prevent this peroxidation, which seems to rule out the involvement of hydroxyl radicals. A more likely explanation, then, for the potentiating effects of xanthine oxidase and $FeSO_4$ on microsomal peroxidation is that xanthine oxidase-generated O_2^-· directly reduces iron and thereby maintains higher levels of the active ferrous form. Reduction of Fe^{3+} by superoxide radicals has been previously reported (21). Since stimulation of peroxidation by xanthine oxidase-generated O_2^-· appears to involve enhancement of the effects of iron rather than conversion to hydroxyl radicals, it seems even more unlikely that NADPH-dependent microsomal peroxidation is initiated by OH·. In addition, we observed that H_2O_2, tested over a hundredfold concentration range, did not increase microsomal lipid peroxidation, even when iron was present (data not shown).

TABLE 1

Effect of SOD and thiourea on xanthine oxidase/Fe^{2+}-induced lipid peroxidation

Microsomes (196 μg protein) were incubated for 30 min with the indicated reagents. The xanthine oxidase system used was shown to produce 8.1 nmol of adrenochrome per minute in the presence of 4 mM epinephrine at 37°C (see Experimental Procedures). Each number represents the mean (±standard deviation) value from three to five experiments.

Condition	Lipid peroxidation
	nmol MDA/mg microsomal protein
Xanthine oxidase (0.53 units)	3.5 ± 1.7
Xanthine oxidase plus SOD (45 units)	4.4 ± 1.8
$FeSO_4$ (1 μM)	2.8 ± 0.5
Xanthine oxidase plus $FeSO_4$	13.6 ± 1.6
Xanthine oxidase plus $FeSO_4$ plus SOD	4.2 ± 1.2
Xanthine oxidase plus $FeSO_4$ plus thiourea (8 mM)	14.1 ± 1.7

The concentration of thiourea which we tested (8 mM) was comparable to that which Lai and Piette (12) reported would inhibit the peroxidation of arachidonic acid in a "Fenton system" containing H_2O_2 and $FeSO_4$. It was assumed that MDA formation in that system was initiated by hydroxyl radicals produced in the Fenton reaction and that scavenging of OH· by thiourea prevented the peroxidation. We have conducted similar experiments, using lipid extracted from liver microsomes (liposomes). As with whole microsomes, liposomal lipid underwent extensive peroxidation when exposed to ferrous iron alone. The addition of H_2O_2, however, at concentrations similar to those employed by Lai and Piette (90–360 μM), did not enhance peroxidation and even inhibited at higher concentrations. Identical results were obtained whether the reaction was initiated by the addition of liposomes or iron. Thus, production of MDA in a Fenton system appears to be due to the direct effect of Fe^{2+} rather than OH·. Peroxidation by Fe^{2+} was also decreased by chelation of iron with an equivalent amount of EDTA, an effect which will be presented more fully later. The addition of 8 mM thiourea caused a 40% decrease in Fe^{2+}-induced liposomal peroxidation in the absence of H_2O_2 which we regard as a general antioxidant effect. Also, neither ethanol nor SOD inhibited MDA formation in this system. These findings, therefore, demonstrate that O_2^-· and OH· are neither initiators nor critical intermediates in liposomal or NADPH-dependent microsomal peroxidation.

Stimulation of liposomal peroxidation by 20 μM ferric iron was only 10% of that produced by the same concentration of ferrous iron. Also, in Fig. 3, the initial rate of microsomal peroxidation produced by ferric iron alone (no NADPH) was more than 20 times lower than that with ferrous iron, and a fourfold stimulation by NADPH was obtained even when the concentration of Fe^{3+} was 40 μM. The slight NADPH-independent activity of ferric iron is probably due to a small amount of Fe^{2+} which may be present in solutions of Fe^{3+}, whereas the addition of NADPH to the microsomal suspensions results in the production of more substantial amounts of the active Fe^{2+} species. Thus promotion of lipid peroxidation by

ferric iron, like that observed in Figs. 1 and 3 and in other laboratories (2–5, 9–11), would appear to be due to the presence of ferrous iron, either added as a contaminant of ferric or produced by the reduction of ferric during the incubation.

We also found that peroxidation of microsomes or liposomes by Fe^{2+} was enhanced by the addition of ascorbate, whereas ascorbate in the absence of iron had no effect. These findings suggest that stimulation of peroxidation by ascorbate, like that induced by NADPH and $O_2^-\cdot$ (produced by xanthine oxidase), is attributable to its ability to reduce iron (22).

Effects of metal complexing agents. It has been previously reported that ADP-chelated iron will promote lipid peroxidation in whole microsomes (2, 6). Similarly, for a system consisting of a purified reductase and isolated liver lipid, an EDTA–iron complex was reported to be required (6). We did not observe any enhancement over free ferrous iron-stimulated peroxidation by either of these complexing agents. Tested over the range of 0.01–2.0 mM, ADP had no effect on microsomal lipid peroxidation, as stimulated by 1.0 to 20 μM ferrous iron in the presence of NADPH, or on the peroxidation of liposomes induced by ferrous iron alone. EDTA, ranging from 8 to 50 μM, did not increase the rate of microsomal or liposomal peroxidation[3] induced by 20 μM Fe^{2+}. Inhibition occurred as the EDTA concentration approached the iron concentration and complete chelation produced complete inhibition of peroxidation. The amount of EDTA required for complete inhibition was higher by about 5 μmol when 200 μM NADPH was present, presumably due to a weakly competitive complexation of iron by NADPH in a form which could still initiate peroxidation. At a higher concentration of Fe^{2+} (100 μM), lipid peroxidation was less[4] than at 20 μM, and under these conditions equimolar EDTA stimulated peroxidation apparently by decreasing free Fe^{2+} to a more effective concentration. Total inhibition occurred, however, when EDTA exceeded iron by 40 μmol.

Further characterization of the role of iron. We have attempted to determine if there is any additional involvement of iron beyond initiation of the lipoperoxidative process. It has been previously suggested that the primary role of iron may be to catalyze decomposition of lipid hydroperoxides or other MDA precursors (7). According to this hypothesis, incubation of microsomes in the absence of iron should result in the formation and accumulation of peroxide intermediates, which would yield a faster rate of peroxidation upon the addition of iron. However, when microsomes were preincubated with NADPH but without iron, the rate of peroxidation which occurred upon the subsequent addition of iron was identical to the rate obtained when no preincubation was performed (data not shown). In addition, Table 2 shows that during incubation with EDTA, no change in the fatty acid content of microsomal lipids occurred. Thus, it appears that the peroxidative sequence requires iron for its initiation. Moreover, stimulation of MDA formation by CCl_4, as reported in the following paper (27), occurs in the absence of iron, which provides strong evidence that iron is not required for the breakdown of MDA precursors or for any steps beyond initiation of peroxidation.

We have also shown that when excess EDTA was added to a rapidly peroxidizing system, there was little or no delay in the cessation of MDA formation. This effect of EDTA must be due to iron chelation rather than any free radical scavenging property since this same amount of EDTA had no effect on CCl_4-induced MDA formation [see the following paper (27)]. Therefore, it appears that the peroxidative sequence leading to MDA formation occurs rapidly, relative to initiation, such that there is virtually no buildup of intermediates whose breakdown to MDA is detectable after chelation of Fe^{2+} by EDTA.

As an additional note, when iron was excluded from the incubations but added just prior to TBA, no colored product was observed. Incubation with iron followed by the addition of a large excess of EDTA prior to the TBA procedure did not affect chromogen formation (data not shown). Therefore, iron is not involved in the reaction of MDA with TBA in our experiments.

Characterization of lipid substrate requirements. The time course of microsomal lipid peroxidation revealed an upper limit to MDA formation of 75–100 nmol MDA/mg

TABLE 2

Association between loss of polyunsaturated fatty acids and MDA formation in liver microsomes

Experimental procedures are given in Experimental Procedures. Microsomes (400–600 μg protein) were incubated for 30 min (except for "not incubated").

Condition	% Total fatty acids					% Maximum MDA formation	% Decrease in 20:4 plus 22:6
	18:2	18:3	20:2	20:4	22:6		
Not incubated	17.0	0.8	1.1	25.6	4.4	ND[a]	0
5 μM EDTA, 100 μM NADPH	17.6	0.7	1.1	26.7	4.6	ND	0
0.5 μM $FeSO_4$, 100 μM NADPH	17.8	0.5	1.0	24.9	3.6	6.7	5.2
10 μM $FeSO_4$, 50 μM NADPH	18.0	1.0	1.3	18.8	3.3	27.5	26.5
2 μM $FeSO_4$, NADPH-generating system	17.9	1.8	1.1	18.3	2.1	31.3	31.8
20 μM $FeSO_4$, 300 μM NADPH	14.7	0.7	0.9	5.0	1.0	71.2	83.1
50 μM $FeSO_4$, 600 μM NADPH	10.7	ND	ND	ND	ND	96.7	100

[a] None detected.

[3] It is unlikely that the lack of stimulation by added EDTA is due to the small amount (less than 10^{-2} μmol) of EDTA present in our microsomal incubations, since the enhancement reported by other investigators (6, 10) was observed at 10^4-fold higher concentrations. In addition, we have prepared microsomes in the absence of EDTA and observed a similar lack of ability of added EDTA to promote free ferrous iron-induced peroxidation.

[4] It is not clear why less peroxidation occurs at high iron concentrations, although an increase in the destruction of free radicals by chain termination reactions, as described by Ingold (23), is suspected.

protein. This cessation of MDA production was not attributable to depletion of NADPH or iron or to inhibition of NADPH–cytochrome *c* reductase and was therefore postulated to represent the total consumption of membrane polyunsaturated fatty acids. Table 2 shows the relationship between the percentage of maximum MDA formation and the percentage decrease in the individual polyunsaturated fatty acids of liver microsomes. Peroxidative membrane decomposition was associated primarily with loss of arachidonic acid (20:4) and docosohexaenoic acid (22:6), consistent with a previous report (4), and total depletion of these two fatty acids corresponded to maximal MDA formation. In fact, the percentage of decrease in 20:4 plus 22:6 very closely paralleled the percentage of maximal MDA formation over a wide range of peroxidizing conditions, thus confirming the reliability of MDA measurement as an indicator of the extent of lipid peroxidation.

DISCUSSION

Our findings strongly support the hypothesis that free ferrous iron is a direct initiator of peroxidation of microsomal or liposomal lipids. The chelating agents ADP and EDTA were not required or stimulatory in our experiments. In previous studies of microsomal peroxidation, ADP was used and its role was suggested as preventing precipitation of iron (6, 10). We found that using freshly prepared solutions of Fe^{3+} or Fe^{2+} precluded any stimulation of peroxidation by ADP. EDTA has been shown to enhance the reduction of Fe^{3+} by purified NADPH–cytochrome *P*-450 reductase and thus EDTA stimulates peroxidation in a liposomal system containing the purified reductase and ferric iron in excess over EDTA (6, 10). We found no EDTA stimulation of NADPH/Fe^{2+}-induced peroxidation of intact microsomes or of Fe^{2+}-induced peroxidation of liposomes. These findings are not consistent with a recently suggested role of EDTA–Fe^{2+} as a propagator of lipid peroxidation (24).

Although we have hypothesized that Fe^{2+} elicits peroxidation by reacting directly with membrane lipids and oxygen, other investigators have proposed that the role of iron is to catalyze the formation of hydroxyl radicals as the ultimate peroxidation-initiating species. In support of this, Lai and Piette (12) observed that the addition of ESR spin-trapping agents which react with hydroxyl radicals would inhibit microsomal peroxidation. However, concentrations of the spin-trap which produced maximal ESR signal intensity in their experiments produced only about 70% inhibition of MDA formation. Thus, the peroxidation they observed cannot be considered entirely dependent on OH· and the possibility remains that the spin-trapping compounds partially inhibited lipid peroxidation by reacting with free radical intermediates other than OH·. In support of this, *p*-nitrosodimethylaniline, an efficient scavenger of radiolytically generated hydroxyl radicals, has been shown to react nonspecifically in several biochemical systems (25). It was concluded from these studies that under biochemical conditions, as opposed to radiolysis or photolysis, no freely diffusible OH· radicals are formed.

It might still be argued, however, that hydroxyl radical-dependent peroxidation was somehow suppressed under the conditions we employed, although we have ruled out the possibility that Tris may exert this effect. On the contrary, the maximal amount of MDA produced in our experiments was over 100-fold greater than the maximal amount of MDA formation observed by Lai and Piette (12), and it is therefore highly unlikely that our experiments were conducted under suboptimal conditions for NADPH/Fe^{2+}-induced peroxidation. Thus, the apparently OH·-independent peroxidative activity we have observed probably represents a major fraction of, if not the total, NADPH-dependent activity.

The mechanism of initiation of lipid peroxidation by ferrous iron is not clear. The involvement of perferryl ion, FeO_2^{2+}, has been previously suggested (9) and seems a likely possibility. In addition, convincing stoichiometric evidence has been recently reported suggesting the formation of a ternary free-radical complex between arachidonic acid, ferrous iron, and oxygen which could result in peroxidation of the hydrocarbon chain (26).

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