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MECHANISM OF OCHRATOXIN A STIMULATED LIPID PEROXIDATION

RABEEA F. OMAR,* BRIAN B. HASINOFF,†‡ FERDINAND MEJILLA* and ANVER D. RAHIMTULA*§

Departments of *Biochemistry, and †Chemistry, and the ‡Faculty of Medicine, Memorial University of Newfoundland, St. John's, Newfoundland, Canada A1B 3X9

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Abstract—Lipid peroxidation, measured as malondialdehyde formation or by oxygen uptake, was stimulated markedly by the mycotoxin ochratoxin A (OTA) in a reconstituted system consisting of phospholipid vesicles, the flavoprotein NADPH–cytochrome P450 reductase, Fe^{3+}, EDTA and NADPH. Deletion of EDTA lowered the extent of lipid peroxidation but did not eliminate it. Fluorometric and spectrophotometric studies demonstrated the formation of a 1:1 Fe^{3+}–OTA complex. The rate of reduction of Fe^{3+} to Fe^{2+} was enhanced markedly in the presence of OTA, and there was a further increase in the rate when EDTA was also included. The data indicate that OTA stimulates lipid peroxidation by complexing Fe^{3+} and facilitating its reduction. Subsequent to oxygen binding, an iron–oxygen complex of undetermined nature initiates lipid peroxidation. Free hydroxyl radicals appear not to participate in lipid peroxidation stimulated by Fe^{3+}–OTA.

Ochratoxin A (OTA‖), a mycotoxin consisting of a 5′-chlorinated 3,4-dihydro-3-methylisocoumarin moiety linked by an amide bond to L-β-phenylalanine (Fig. 1), is produced by some species of the genera *Aspergillus* and *Penicillium*. The occurrence of OTA in food and feed is widespread [1], and it is known to be highly toxic to animals [2–4]. The main pathological changes associated with OTA toxicity are kidney and liver damage [5, 6]. Alterations in a variety of biochemical and immunological parameters have also been observed following OTA administration [7–10]. In addition, dietary feeding of OTA has been shown to induce renal adenomas and hepatocellular carcinomas in mice [11]. OTA did not produce genetic or related effects in a variety of *in vitro* short-term tests [12, 13], but it has been shown to induce DNA single-strand breaks in liver, kidney and spleen of mice [14].

Recently, we showed that addition of OTA to rat liver or kidney microsomes or administration of OTA to rats enhances lipid peroxidation *in vitro* and *in vivo* respectively [15]. The peroxidation of polyunsaturated fatty acids present in membrane lipids has been proposed as a mechanism by which a wide and ever-increasing range of compounds produce structural tissue injury [see Refs. 16 and 17 for reviews]. We have now examined the mechanism by which OTA stimulates lipid peroxidation using a reconstituted system consisting of microsomal phospholipid, the flavoprotein NADPH–cytochrome P450 reductase (Fp) and iron ions. Our results indicate that OTA enhances lipid peroxidation primarily by chelating ferric ions (Fe^{3+}) and facilitating their reduction to ferrous ions (Fe^{2+}).

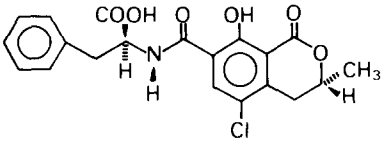

Fig. 1. Structure of ochratoxin A.

MATERIALS AND METHODS

Chemicals. 2′,5′-ADP agarose, bathophenanthroline disulfonic acid, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), catalase, CHAPS, cholic acid, cytochrome *c*, EDTA, Lubrol PX, NADPH, OTA, superoxide dismutase and 2-thiobarbituric acid (TBA) were purchased from the Sigma Chemical Co. (St. Louis, MO). Anhydrous ferric chloride, ferric nitrate and ferrous chloride were obtained from BDH Chemicals, Dartmouth, Nova Scotia. All other chemicals were of the highest grade commercially available.

Preparation of microsomes. Male Sprague–Dawley rats (200–220 g) were obtained from Canadian Hybrid Farms, Halifax, Nova Scotia, and were allowed free access to standard laboratory rat chow and water. Untreated rats or rats pretreated with sodium phenobarbital (PB; 0.1% PB in drinking water for 5 days) were starved overnight prior to use. Liver microsomes were isolated by differential centrifugation as described earlier [18].

Purification of NADPH–cytochrome P450 reductase. The flavoprotein NADPH–cytochrome

§ Address all correspondence to: Dr. Anver D. Rahimtula, Department of Biochemistry, Memorial University of Newfoundland, St. John's, Newfoundland, Canada A1B 3X9.

‖ Abbreviations: BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; BPS, bathophenanthroline disulfonic acid; CHAPS, 3-[(cholamidopropyl)-dimethylammonio]-1-propanesulfonate; MDA, malondialdehyde; OTA, ochratoxin A; PB, sodium phenobarbital; SOD, superoxide dismutase; TBA, 2-thiobarbituric acid; and TCA, trichloroacetic acid.

P450 reductase (Fp) was purified as described by Murray Ardies *et al.* [19] from liver microsomes isolated from PB-pretreated rats. Briefly, this involved treating microsomes with the detergent CHAPS in combination with protamine sulfate followed by centrifugation. Fp was solubilized from the CHAPS-insoluble pellet by a combination of the detergents sodium cholate and Lubrol PX and purified to homogeneity by 2′,5′-ADP agarose affinity chromatography. The purified Fp showed a single band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and had a specific activity of 18,000 units/mg protein at 22° using cytochrome *c* as the electron acceptor [20]. One unit of enzyme activity is defined as that amount which catalyzes the reduction of 1 nmol cytochrome *c*/min.

Preparation of phospholipid vesicles. Total lipid was extracted from untreated rat liver microsomes by the method of Folch *et al.* [21] with care being taken to flush all solvents with nitrogen and to perform all operations under nitrogen at 0–4° to minimize auto-oxidation of polyunsaturated lipids. The extracted lipid in chloroform:methanol (2:1) was stored under nitrogen in aliquots at −80°. Total lipid phosphorous was determined as described by Bartlett [22]. Phospholipid vesicles were prepared fresh daily by sonication of the extracted lipid under anaerobic conditions as described in Ref. 23. Briefly, an aliquot of the phospholipid solution was evaporated to dryness in a plastic tube under nitrogen, and nitrogen-saturated Tris–HCl buffer (0.25 M; pH 6.8) was added to give a final lipid phosphorous concentration of 10 μmol/mL. The tube was flushed with nitrogen, capped, and placed in a glass beaker filled with a mixture of ice and water. Phospholipid vesicles were obtained by placing the probe of a Branson sonifier (model W185) in the beaker and applying a power of 50 W for 5 min.

Lipid peroxidation assays. Incubations were carried out in triplicate at 37° in 0.25 M Tris–HCl buffer (pH 6.8)/0.25 M NaCl. The complete system contained Fp, phospholipid vesicles, Fe^{3+}, OTA, EDTA and NADPH. Final concentrations of the various components are given in the figure and table legends. Lipid peroxidation was initiated by addition of NADPH and terminated by transferring 0.5-mL aliquots of the reaction mix into tubes containing 50 μL of 2% BHT in ethanol and 500 μL of 30% trichloroacetic acid (TCA). The tubes were heated in a boiling water bath for 15 min, cooled, and centrifuged, and the absorbance of the MDA–TBA adduct was read at 535 nm [24]. Various agents, when included in the incubation mix, were added before initiation of lipid peroxidation.

Spectrophotometric measurements. Spectrophotometric measurements were conducted at 25° on either a Shimadzu UV-260 or a Perkin–Elmer Lambda 3B double beam spectrophotometer in 1 cm cells. Fluorescence measurements were conducted on a Perkin–Elmer LS-5 spectrofluorimeter in 1 cm cells.

Reduction of Fe^{3+} *to* Fe^{2+}. Reduction of Fe^{3+} to Fe^{2+} was measured spectrophotometrically by recording the time-dependent increase in absorbance at 535 nm ($E = 22.14\ mM^{-1}\ cm^{-1}$) due to formation of the colored water-soluble bathophenanthroline disulfonic acid–Fe^{2+} complex [25]. The reaction mixture contained per mL: 3.2 units Fp, 110 μM Fe^{3+}, 10 μM EDTA, 250 μM OTA, 400 μM BPS and 200 μM NADPH. The reaction was started by addition of NADPH.

Oxygen uptake studies. Oxygen uptake was measured polarographically with a Clark electrode [26]. The reaction mixture contained in a total volume of 1.5 mL: 0.25 M Tris–HCl (pH 6.8), 4.8 units Fp, phospholipid vesicles (1.5 μmol lipid P), 110 μM Fe^{3+}, 25 μM EDTA, and various concentrations of OTA (0–1000 μM). Appropriate controls were performed omitting one or the other of the various components. Rates of oxygen consumption showed significant variation from day to day presumably due to the free radical nature of the reaction. However, the relative rates of oxygen uptake under the various conditions were always consistent although the absolute values differed.

RESULTS

Inclusion of OTA in a reconstituted enzyme system markedly enhanced the rate of lipid peroxidation. Figure 2 shows the time–course of this lipid peroxidation. The rate of MDA formation was approximately linear but showed a slight lag phase. About 9 nmol of MDA was formed at the end of 60 min when the complete system consisting of Fp, phospholipid, Fe^{3+}, EDTA, OTA and NADPH was used (Fig. 2). Fe^{3+} was essential since its absence led to no MDA formation (Fig. 2B). Very little MDA was formed (<1 nmol) at the end of 1 hr in the absence of OTA (Fig. 2B). Deletion of EDTA, Fp or NADPH from the incubation system resulted in lower rates of MDA formation (~3.5 to 5.0 nmol/hr). As expected, the antioxidant BHA completely inhibited lipid peroxidation (Table 1). However, the hydroxyl radical scavengers mannitol and sodium formate as well as catalase, which decomposes hydrogen peroxide, were without effect, suggesting that these species do not participate in lipid peroxidation (Table 1). However, a slight inhibition (23%) was observed in the presence of superoxide dismutase. The rate of MDA formation increased with increasing OTA concentration (Fig. 3). Concentrations higher than 1 mM OTA were not tested due to insolubility. Results essentially similar to those in Figs. 2 and 3 were obtained when oxygen uptake instead of MDA formation was measured as an index of lipid peroxidation (Figs. 4 and 5). Since EDTA was required for maximum lipid peroxidation (Figs. 2B and 4), we examined the effect of varying the EDTA concentration on MDA levels keeping the concentration of Fe^{3+} fixed at 110 μM. A broad bell-shaped curve was obtained with maximum stimulation of 4- to 5-fold at an EDTA concentration of 25 μM (Fig. 6). However, the amount of EDTA required appeared not to be critical since 5 μM EDTA was almost as effective. Increasing the EDTA concentration to 75 μM and beyond led to a total inhibition of lipid peroxidation presumably due to excessive Fe^{3+} chelation. The effect of Fp concentration on the rate of MDA formation is shown in Fig. 7. In the absence of Fp, a lower rate of MDA formation was observed, indicating that NADPH

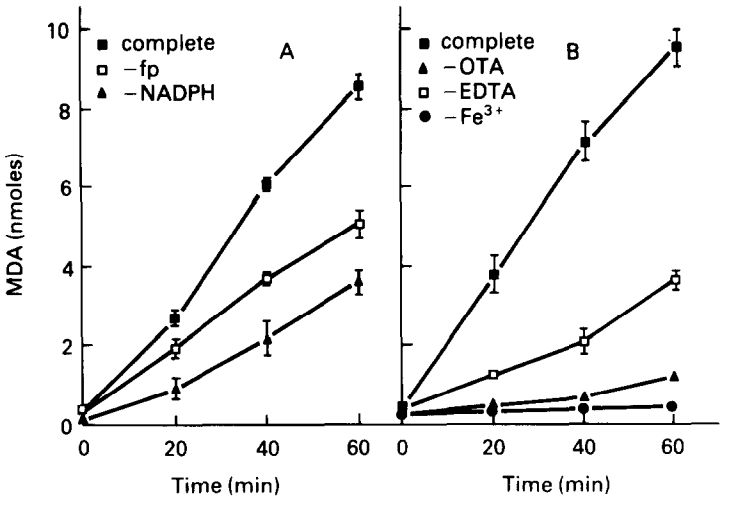

Fig. 2. Effects of various components of the reconstituted system on OTA stimulated MDA formation. Incubations were carried out at 37° for 0, 20, 40 and 60 min in 0.25 M Tris–HCl buffer (pH 6.8)/0.25 M NaCl. The complete system contained per mL: phospholipid vesicles (1 μmol P), 500 nmol OTA, 50 nmol EDTA, 110 nmol Fe^{3+}, 177 ng Fp (3.2 units) and 200 nmol NADPH. Other details are as described in Materials and Methods. The effect of omitting Fp or NADPH is shown in panel A, while the omission of OTA, EDTA or Fe^{3+} is shown in B. Experiments in A and B were carried out separately; each point is the mean ± SD of triplicate incubations from one experiment typical of two.

Table 1. Effects of various agents on OTA-stimulated lipid peroxidation

Addition to system	MDA formed* (nmol)
None	10.47 ± 0.71 (100)
SOD (6.0 μg/mL)	8.10 ± 0.42 (77)
Catalase (36 μg/mL)	11.20 ± 0.70 (108)
Sodium formate (11 mM)	11.81 ± 0.81 (113)
Mannitol (11 mM)	13.74 ± 0.94 (131)
BHA (50 μM)	0.73 ± 0.01 (7)

* Incubations were carried out in triplicate for 40 min at 37° in 0.25 M Tris–HCl buffer, pH 6.8/0.25 M NaCl and contained (per mL): phospholipid vesicles (1 μmol P), 177 ng Fp (3.2 units), 25 nmol EDTA, 110 nmol Fe^{3+}, 200 nmol NADPH and 500 nmol OTA. All concentrations indicated are final concentrations in the reaction medium. MDA values obtained are means ± SD of triplicate incubations from one experiment typical of three. The numbers in parentheses represent percentage activity relative to "None" as 100%.

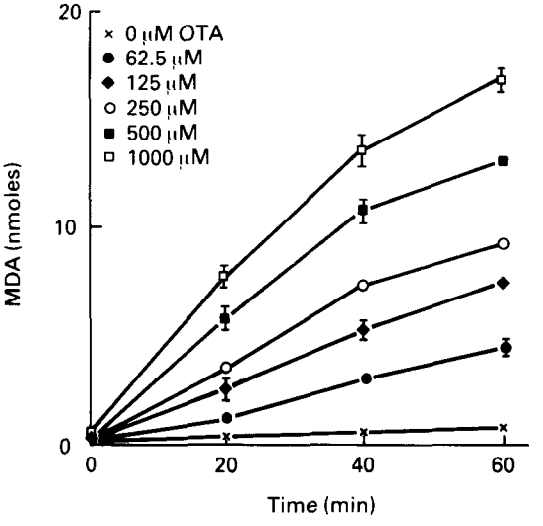

Fig. 3. Effect of OTA concentration on MDA formation. Incubations were carried out at 37° for 20, 40 and 60 min in 0.25 M Tris–HCl buffer, pH 6.8/0.25 M NaCl and contained per mL: phospholipid vesicles (1 μmol P) 25 nmol EDTA, 110 nmol Fe^{3+}, 200 nmol NADPH, 177 ng Fp (3.2 units) and various amounts of OTA (0–1000 μM). Each point is the mean ± SD of triplicate incubations from one experiment typical of three.

is directly able to reduce Fe^{3+} to Fe^{2+}. However, addition of Fp substantially increased the rate of MDA formation especially at earlier time points. Thus, after 5 min, the MDA level in the absence of Fp was 0.22 nmol, and this increased 3-fold (0.60 nmol), 4.5-fold (0.99 nmol) and 11-fold (2.52 nmol) in the presence of 3.2 units, 6.4 units and 16.0 units of Fp respectively (Fig. 7). After 60 min, the differences were much less and increases of 42%, 73% and 110% over the basal rate were observed in the presence of 3.2 units, 6.4 units and 16.0 units of Fp respectively (Fig. 7). The pH optimum was found to be around neutrality with acidic (pH 6.0) or alkaline (pH 9.0) conditions being substantially inhibitory. Thus, all incubations were carried out at a pH of 6.8.

Reduction of Fe^{3+} to Fe^{2+} was followed over a 15-min period by recording the increase in absorbance at 535 nm due to formation of the Fe^{2+}–BPS complex (Fig. 8). No reduction of Fe^{3+} was observed in the absence of NADPH, whereas the maximum rate of reduction was observed in the complete system which included both OTA and EDTA (11.7 nmol/min).

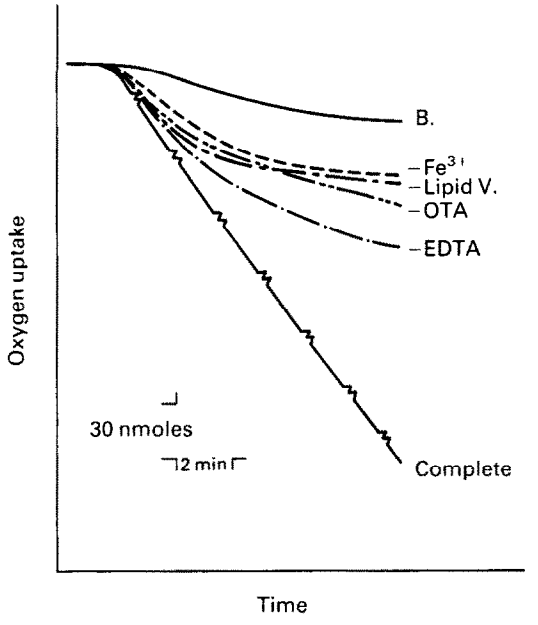

Fig. 4. Effect of various components on OTA stimulated oxygen uptake. Oxygen consumption was measured polarographically with a Clark electrode. The reaction was carried out at 37° in 0.25 M Tris–HCl buffer, pH 6.8/0.25 M NaCl and contained per mL: phospholipid vesicles (1 μmol P), 25 nmol EDTA, 110 nmol Fe^{3+}, 200 nmol NADPH, 177 ng Fp (3.2 units) and 250 nmol OTA. The results represent one experiment typical of two.

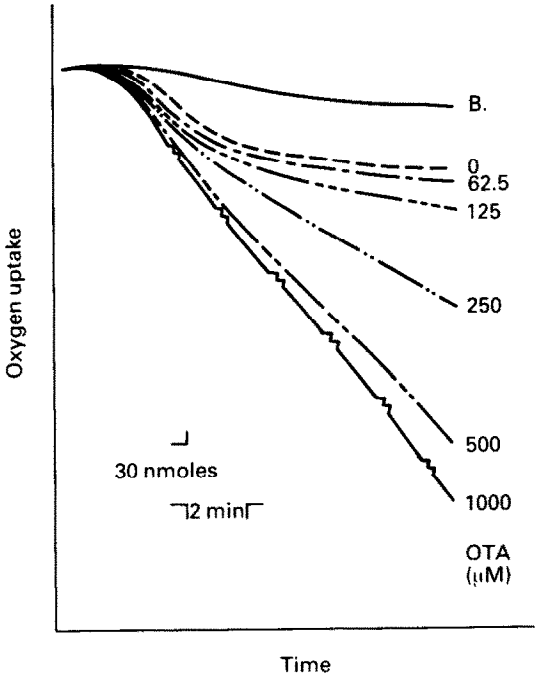

Fig. 5. Effect of OTA concentration on oxygen uptake. Oxygen consumption was measured polarographically with a Clark electrode. The reaction was carried out at 37° in 0.25 M Tris–HCl buffer, pH 6.8/0.25 M NaCl and contained per mL: phospholipid vesicles (1 μmol P), 25 nmol EDTA, 110 nmol Fe^{3+}, 200 nmol NADPH, 177 ng Fp (3.2 units) and various amounts of OTA (0–1000 μM). The results represent one experiment typical of two.

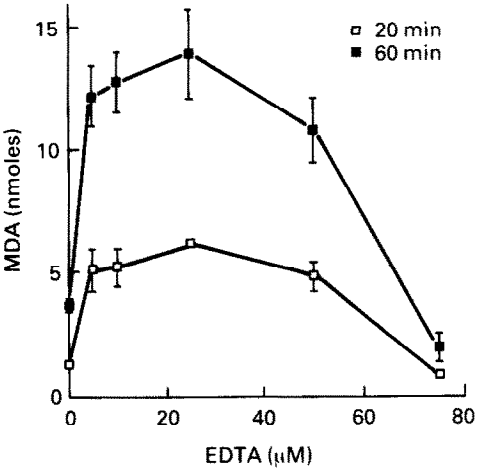

Fig. 6. Effect of EDTA concentration on OTA stimulated lipid peroxidation. Incubations were carried out at 37° for 20 and 60 min in 0.25 M Tris–HCl buffer, pH 6.8/0.25 M NaCl and contained per mL: phospholipid vesicles (1 μmol P) 500 nmol OTA, 110 nmol Fe^{3+}, 200 nmol NADPH, 177 ng Fp (3.2 units) and various amounts of EDTA (0–75 μM). Each point is the mean ± SD of triplicate incubations from one experiment typical of three.

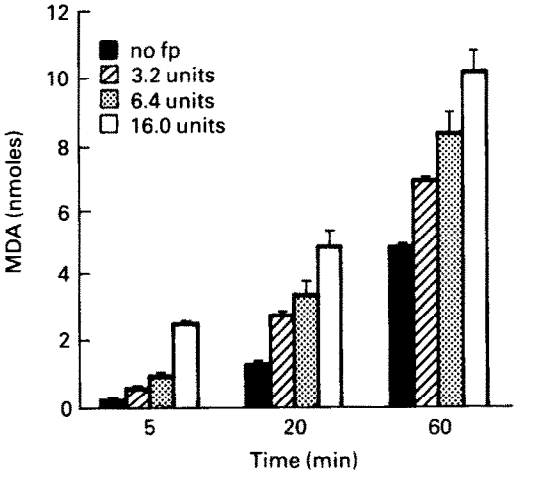

Fig. 7. Effect of Fp concentration on OTA stimulated lipid peroxidation. Incubations were carried out at 37° for 5, 20 and 60 min in 0.25 M Tris–HCl buffer, pH 6.8/0.25 M NaCl and contained per mL: phospholipid vesicles (1 μmol P) 250 nmol OTA, 25 nmol EDTA, 110 nmol Fe^{3+}, 200 nmol NADPH and various amounts of Fp (0–16 units; 0–885 ng). Each bar represents the mean ± SD of triplicate incubations from one experiment typical of two.

Omission of EDTA led to a 21% reduction in the rate of Fe^{2+} formation (9.2 nmol/min), whereas deletion of OTA reduced the rate by 70% (3.5 nmol/min). In the absence of both EDTA and OTA, the rate of Fe^{2+} formation was only 2.7 nmol/min (Fig. 8). The data clearly show that OTA by itself substantially increased the rate of Fe^{3+} reduction and that addition of EDTA further enhanced this effect.

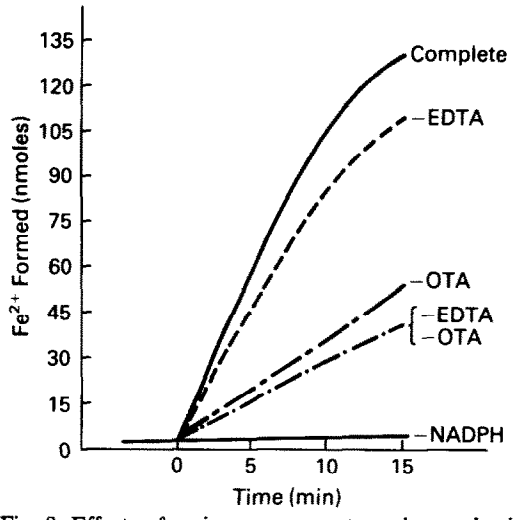

Fig. 8. Effects of various components on iron reduction. Incubations were carried out at 37° in 0.25 M Tris–HCl buffer, pH 6.8/0.25 M NaCl in a 3-mL spectrophotometer cuvette and contained per mL: 177 ng Fp (3.2 units), 200 nmol NADPH, 250 nmol OTA, 10 nmol EDTA, 110 nmol Fe^{3+} and 400 nmol bathophenanthroline disulfonic acid. The results represent one experiment typical of two.

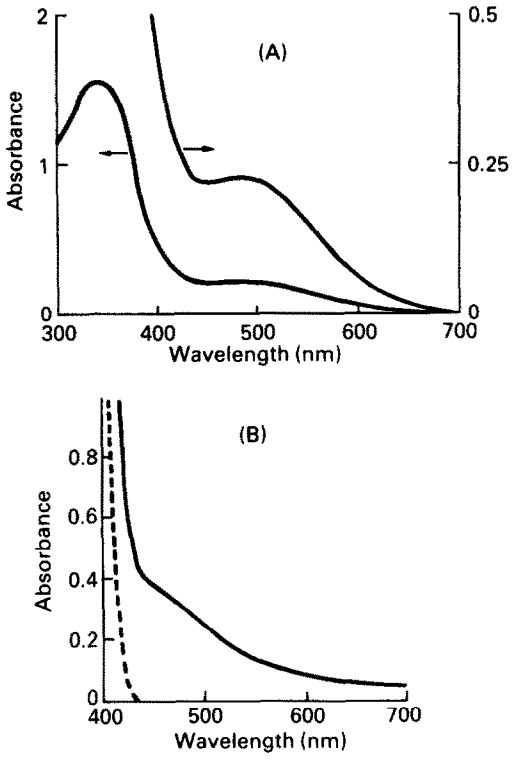

Fig. 9. (A) Spectrum of the Fe^{3+}–OTA complex formed in methanol. The concentrations of Fe^{3+} and OTA were both 200 μM. (B) Spectrum of Fe^{3+}–OTA complex in Tris buffer. $FeCl_3$ and OTA, both dissolved in methanol, were mixed together and then diluted into the buffer (pH 8.6). The Fe^{3+} and the OTA concentrations were, after dilution, 330 and 1000 μM respectively. A small amount of precipitate was observed in the cell after dilution of the Fe^{3+}–OTA complex into the buffer. The broken line (---) is the spectrum of OTA alone and the continuous line (——) that of the Fe^{3+}–OTA complex.

When anhydrous $FeCl_3$, dissolved in methanol, was added to OTA in methanol, a reddish–brown complex was formed with λ_{max} at 342 and 483 nm (Fig. 9A). The spectrum of the Fe^{3+}–OTA complex in aqueous pH 8.6 Tris buffer (Fig. 9B) was similar to that found in methanol except that the peak at 483 nm was converted into a shoulder. The spectrophotometric titration of 190 μM OTA with $FeCl_3$ (Fig. 10) indicated that in methanol a 1:1 Fe^{3+}–OTA complex was formed, though the degree of curvature the plot exhibited suggested that the complex may not be completely formed under these conditions. Attempts to perform similar spectrophotometric titrations in either pH 7.0 or 8.6 aqueous Tris buffers were unsuccessful due to insolubility problems with both OTA and the Fe^{3+}–OTA complex.

The binding of Fe^{3+} to OTA in methanol was also followed fluorometrically (Ex_{max} 340 nm; Em_{max} 465 nm) (Fig. 11, A and B). Preliminary experiments at a constant OTA concentration of 5 μM and in the presence of increasing amounts of $FeCl_3$ showed that the fluorescence was about one-half quenched at a 6-fold excess of $FeCl_3$ (Fig. 11B) and completely quenched at a 40-fold excess of $FeCl_3$. Assuming an equilibrium of the type:

$$Fe^{3+} + HL \rightleftharpoons FeL^{2+} + H^+$$

$$K = \frac{[FeL^{2+}][H^+]}{[Fe^{3+}][HL]}$$

it can be calculated from the titration data of Fig. 11A, which was carried out in the presence of a 100 μM HCl, and from an additional titration conducted under the same conditions but at a constant $FeCl_3$ of 1 μM (data not shown), that K has a value of 19 ± 6 (N = 11) (L^- and HL are the phenolate

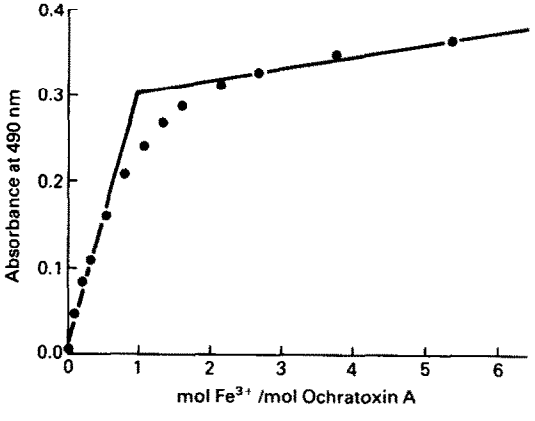

Fig. 10. Titration of OTA by $FeCl_3$. Spectrophotometric titration in methanol at 490 nm of 190 μM OTA by $FeCl_3$. The intersection of the two least squares calculated straight lines (through the lowest 5 and the highest 3 data points, respectively) intersect at a mole ratio of 1.0, indicating a 1:1 complex is formed under these conditions.

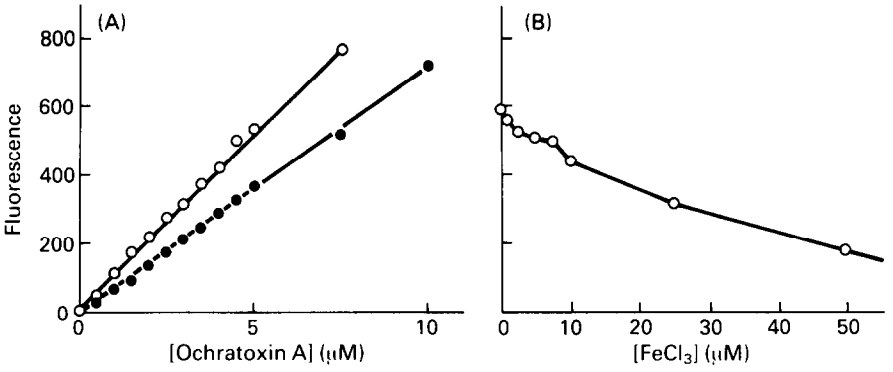

Fig. 11. Quenching of OTA fluorescence by Fe^{3+}. (A) Change in fluorescence (in arbitrary units) when OTA was added to a solution containing no (○) and 5 μM (●) $FeCl_3$ respectively. The solvent was methanolic HCl (100 μM). (B) Change in fluorescence when $FeCl_3$ (in 10 mM HCl) was added to 5 μM OTA in methanol. Addition of 10 mM HCl alone did not alter the fluorescence of OTA.

anion and phenol forms of OTA respectively). To calculate the related association constant, K_{ass} for

$$Fe^{3+} + L^- \rightleftharpoons FeL^{2+}$$

$$K_{ass} = \frac{FeL^{2+}}{[Fe^{3+}][L^-]}$$

it is necessary to know the pK_a of *HL* (assumed to be that of the phenolic group). While this value is unknown in methanol, assuming that it is the same as it is in water (pK_a 7.05 [27]), K_{ass} can then be calculated to be approximately $2 \times 10^8\ M^{-1}$. While this K_{ass} can be considered to be only an order-of-magnitude estimate of the value that might obtain in aqueous solution, its magnitude does indicate that OTA is capable of forming a relatively strong complex with Fe^{3+}, even in the micromolar concentration range.

DISCUSSION

Membrane lipid peroxidation is an important part of oxidative tissue injury and can be an effect as well as a cause of reactions culminating in cytotoxicity [28]. A wide and ever-increasing range of compounds has been shown to induce lipid peroxidation both *in vitro* and *in vivo* (see Refs. 16 and 17 for reviews). Xenobiotics may enhance lipid peroxidation in one of several ways. Haloalkanes like carbon tetrachloride initiate lipid peroxidation subsequent to cytochrome P450-dependent reductive activation to the trichloromethyl radical [29]. A variety of other agents like paraquat [30], mitomycin *c* and nitrofurantoin [31] can redox cycle resulting in oxygen radical formation which can stimulate lipid peroxidation. Yet other compounds like acetaminophen and bromobenzene initiate lipid peroxidation through depletion of cellular glutathione [32]. Finally, compounds like ADP can chelate iron and the resulting ADP–iron complex can undergo enhanced redox cycling thus stimulating lipid peroxidation [33]. By using a reconstituted system, we were able to show that OTA stimulates lipid peroxidation by the last-mentioned mechanism. Consistent with this is the observation that HPLC analysis did not reveal any appreciable biotransformation of OTA (data not shown).

Our results clearly indicate that a reconstituted system consisting of phospholipid vesicles, purified reductase, NADPH, Fe^{3+}, OTA and EDTA is efficient in carrying out lipid peroxidation measured either as MDA formation (Fig. 2) or oxygen uptake (Fig. 4). Omission of OTA gave rise to very little peroxidation (<10%), whereas deletion of EDTA reduced the extent of MDA formation by 70% (Fig. 2). Some oxygen uptake was observed in the absence of phospholipid (Fig. 4). This is likely due to the ability of NADPH–cytochrome P450 reductase to reduce molecular oxygen to superoxide anion [34]. Pederson and Aust [35] first characterized such a lipid peroxidation system using ADP instead of the OTA used here. In their reconstituted system, EDTA was required as well since no peroxidation occurred in its absence [35]. Fe^{3+} rapidly precipitates out of neutral aerobic solutions to form insoluble ferric hydroxides and it was recognized some time ago that complexing iron with ligands such as ADP and EDTA overcomes this problem [33]. OTA could thus be playing a similar role. In support of this, we have provided spectrophotometric and fluorometric evidence for the formation of an Fe^{3+}–OTA complex (Figs. 9–11).

It is generally accepted [16, 17, 33] that reduction of the various Fe^{3+} chelates capable of initiating lipid peroxidation proceeds via NADPH–cytochrome P450 reductase (Fp). The ability of Fp to reduce Fe^{3+}–EDTA but not Fe^{3+}–ADP [36] is in agreement with the iron chelate requirements of the reconstituted system used by Pederson and Aust [35]. In our case, EDTA markedly enhanced the rate of OTA-dependent lipid peroxidation but was not absolutely essential (Fig. 2). This is consistent with our finding that Fp was able to reduce Fe^{3+}–OTA, suggesting that in microsomes another carrier could mediate the transfer of electrons from NADPH to Fe^{3+}–ADP [33]. This has led to the demonstration that in a reconstituted system, cytochrome P450 (normally present in liver microsomal membranes) can replace Fe^{3+}–EDTA in stimulating lipid peroxidation [37, 38]. OTA-stimulated microsomal lipid

peroxidation also does not require EDTA [15]; thus, cytochrome P450 could play a similar role in our reconstituted system. Preliminary studies, using cytochrome P450 isolated from livers of PB-pretreated rats, indicate that such is the case.

Data presented in Figs. 9–11 demonstrate the binding of Fe^{3+} to OTA, both in methanol and in aqueous solution. The cardiotoxic anthracyclic quinone antitumor drug doxorubicin also has a phenolic group beta to a carbonyl group and likewise forms a strong complex with Fe^{3+} [39]. An iron-based oxidative stress produced through an enzymatic reductive activation is thought to be partly responsible for doxorubicin-induced cardiotoxicity.

Ernster, Hochstein and co-workers [40] have investigated the role of iron and iron chelators in the initiation of microsomal lipid peroxidation. Their studies showed that an Fe^{3+} chelate in order to enzymatically initiate microsomal lipid peroxidation has to fulfill three criteria: (i) reducibility by NADPH; (ii) reactivity of the Fe^{2+} chelate with oxygen; and (iii) formation of a relatively stable perferryl radical. They demonstrated reduction of the various Fe^{3+} chelates by NADPH oxidation. We have shown that Fe^{3+}–OTA is reduced by measuring the formation of Fe^{2+} with bathophenanthroline disulfonate (Fig. 8). They demonstrated interaction of the various Fe^{2+} chelates with oxygen and formation of relatively stable perferryl chelates with the use of an oxygen electrode [40]. These tests were performed in the absence of microsomes, simply by adding Fe^{2+} to the respective chelators in a buffer (0.1 M Tris–HCl, pH 7.5) and recording oxygen consumption. The slow uptake of oxygen (with ADP, ATP, oxalate or malonate) as opposed to no uptake (with cyanide or *o*-phenanthroline) or instantaneous uptake (with EDTA or pyrophosphate) was interpreted as interaction of the Fe^{2+} chelate with oxygen and formation of a relatively stable perferryl complex. We have repeated this experiment with OTA and Fe^{2+} and have found it to interact with oxygen slowly (data not shown), thus fulfilling the three criteria set forth by Ernster and co-workers [40].

In our studies, we did not attempt to distinguish between the various species involved in initiating lipid peroxidation. The precise nature of the initiating species is not known and is currently the focus of active research. Several workers [40–42] have implicated the perferryl ion as the initiating species; however, its ability to extract a methylene hydrogen has been questioned due to its poor reactivity [43]. Recently, Koppenol [44] has proposed that the more reactive ferryl ion be considered as an alternative. Aust and co-workers [45] have suggested that a Fe^{3+}–O_2–Fe^{2+} complex may be the initiating species but this has been disputed recently [46]. Hydroxyl radicals can initiate lipid peroxidation in homogeneous reaction systems [47]; however, the use of scavengers has unequivocally failed to show any significant involvement of hydroxyl radicals in microsomal or liposomal peroxidation systems [48]. Our results, showing the lack of inhibitory effect of hydroxyl radical scavengers (Table 1), are consistent with earlier studies [48]. The slight inhibition observed in the presence of SOD (23%) could be due to its metal binding ability and/or its ability to inhibit the superoxide-dependent reduction of the Fe^{3+}–OTA complex.

Sugioka *et al.* [49] examined the Adriamycin® stimulated ADP–Fe^{3+} dependent unsaturated phospholipid decomposition in a model system that included microsomal phospholipid, NADPH and Fp. They provided evidence that the ternary complex of Fe^{2+}–ADP–Adriamycin® was the active species. For a variety of reasons, we do not feel that formation of a similar EDTA–Fe^{2+}–OTA ternary complex occurs in our system since the two are not comparable. EDTA binds Fe^{3+} many times more strongly than OTA ($K_{EDTA} \simeq 10^{25}$). Thus, the equilibrium for the reaction.

$$Fe^{3+}\text{–OTA} + \text{EDTA} \rightleftharpoons Fe^{3+}\text{–EDTA} + \text{OTA}$$

lies very far to the right. Also, Fe^{3+} is hexa-coordinate with EDTA and could not go octa-coordinate to form a Fe^{3+}–OTA–EDTA complex. In contrast, ADP forms a weak complex with Fe^{3+}, thus permitting the formation of a Fe^{3+}–ADP–Adriamycin® complex.

In conclusion, our studies indicate that OTA stimulates lipid peroxidation by complexing with iron and facilitating its reduction. The extent of OTA-dependent lipid peroxidation *in vivo* and its role in the toxicity of OTA remain to be determined.

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